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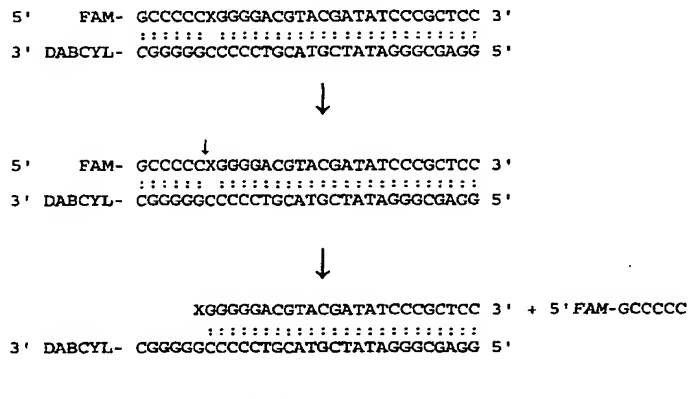

X = an artificial 'abasic' site, (e.g. Tetrahydrofuran)

FAM = quenched fluoroscein fluor.

FAM = excitable fluoroscein fluor.

DABCYL = quenching molecule

↓ = scission of the phosphodiester bond

(57) Abstract: The present invention provides a polynucleotide having a double stranded portion which is interrupted by at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair, the molecule further having attached thereto a fluorescent moiety and a quenching moiety which quenches the fluorescence of the fluorescent moiety. The invention further provides assays and methods using said polynucleotides, and uses of agents identifiable using said polynucleotides which inhibit DNA modifying enzymes.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS AND MATERIALS

The present invention relates to polynucleotides and their use in methods for assaying the activity of a DNA modifying enzyme, and the use of the assays for identifying compounds which modulate enzyme activity.

Enzymes which modify nucleic acid strands are well known in molecular biology. Some of these enzymes require single stranded nucleic acid as a substrate, and others are selective for double stranded nucleic acid.

The action of some enzymes which require the nucleic acid substrate to be double stranded can produce modification on both of the strands. An example of these enzymes are the restriction endonucleases, which recognise specific, and usually palindromic, base-pair sequences in double-helical DNA and cleave the phosphodiester backbone of both strands.

However, some enzymes which require double stranded nucleic acid as a substrate may produce a modification in only one of the two strands. Such enzymes include the mammalian DNA "repair" enzymes, which recognise residues which do not participate in an A-T or G-C base pair. Examples are DNA glycosylases which recognise damaged bases on either strand (discussed in Krokan *et al* (1997) *Biochem. J.* 325:1-16) , AP endonucleases which recognise an apurinic/apyrimidinic (AP) site (ie, a baseless or abasic site) in either strand of the DNA, and XPG-like enzymes, such as the Nucleotide Excision Repair nucleases which include the XPF-ERCC1 complex (Wood (1997) *J. Biol. Chem.* 272:23465-23468), which recognise sections of non-complementary bases.

One of the features specifically recognised by enzymes with DNA glycosylase activity is the "damaged" base 7,8-dihydro-8-oxoguanine (8-oxoguanine), which is an important mutagenic lesion. The Nth1 and OGG1 proteins are examples of DNA glycosylases that can remove oxidative lesions such as 8-oxoguanine from DNA (Marenstein *et al* (2001) *J. Biol. Chem.* 276;21242-9)

The apurinic/apyrimidinic (AP) site recognised by AP endonucleases is one of the most common lesions in DNA. An AP site is one which has an intact phosphodiester bond and attached sugar residue, but which lacks a purine or pyrimidine base residue and is therefore abasic. AP sites can arise either through spontaneous hydrolysis by the action of reactive oxygen species (see Lindahl (1990) *Mutat. Res.* 238:305-311 and Lindahl (1993) *Nature* 362:709-715), by the removal of modified bases by DNA glycosylases (Loeb (1985) *Cell* 40:483-4) within the base excision repair pathway, or as a result of base damage generated by DNA damaging drugs or ionising radiation. These abasic sites disrupt DNA replication and are highly mutagenic if not repaired. Repair by an AP endonuclease is generally initiated by cleavage 5' to the lesion. AP endonucleases are classified into two families according to their homology to *E. coli* endonucleases: exonuclease III (xth) and endonuclease IV (nfo). The first of these families derives from organisms across several phyla, including ExoIII (*E. coli*), Exo A (*Streptococcus pneumoniae*), Rrp 1 (*Drosophila melanogaster*), Arp (*Arabidopsis thaliana*), Apn2 (*S. cerevisiae*), APEX (mouse), BAP1 (bovine), rAPE (rat) and chAPE1 (hamster). The main AP endonuclease in human cells is HAP1 (also known as Ape1 and Ref-1), which is a multi-functional enzyme that, as well as being involved in the repair of AP sites, functions as a redox factor, maintaining numerous transcription factors in an active state (for a review see Evans, A.R. *et al.* (2000) *Mutation Research*

461, 83-108). HAP1 exhibits strong AP hydrolytic activity, along with the other enzymes having homology to Exo III. Repair catalysed by HAP1 is generally initiated by endonucleolytic cleavage 5' to the abasic site. Furthermore, HAP1 is a multifunctional enzyme that also possesses 3' phosphodiesterase activity.

Where a damage occurs in DNA, a bubble of non-A-T or G-C base-paired, single-stranded, DNA may be created within duplex DNA by helicase activity. Such a bubble may also be produced by a stretch of residues which are not complementary in terms of A-T or G-C base pairing to the residues on the other strand . Such "bubbles" of single-strandedness may represent bulky, helix-distorting lesions. Some DNA repair enzymes recognise this lack of double strandedness in DNA and cleave the DNA phosphodiester backbone 5' to the non-double stranded base. Well-known examples of such an enzyme are human XPG and the XPF-ERCC1 complex, which exhibit endonuclease activity. XPF-ERCC1 cleaves near the border between single-stranded and duplex DNA when the single strand has a polarity 5' to 3' moving away from the border, whereas junctions between duplex and single-stranded DNA are cleaved with the opposite polarity by the XPG protein (Wood (1997) *J. Biol. Chem.* 272:23465-23469).

Base excision repair is a critical mechanism for removing mutagenic and/or cytotoxic lesions from the DNA. Hence, the role played by DNA repair enzymes means that they are potentially important targets for new cancer therapies.

US 6,190,661 discloses that reduction of HAP1 activity may be used to (a) treat HAP1 related premalignant or malignant conditions, (b) induce apoptosis in a cell and (c) enhance the sensitivity of a HAP1 related

premalignant or malignant tumour cell to chemotherapy, radiotherapy or gene therapy. The method for reducing HAP1 activity described in US 6,190,661 includes inhibiting function or expression of HAP1. However, US 6,190,661 does not disclose polynucleotides which can be used in a method for identifying agents which inhibit the function of HAP1, nor does it disclose low molecular weight inhibitors of HAP1.

WO 99/35288 discloses assays for detecting hydrolysis of nucleotide bonds in polynucleotides by nucleases such as thermonuclease or *Staphylococcus* nuclease. US 5,763,181 discloses a method of detecting the activity of a nuclease such as DNase I or *Bam*HI using a double stranded oligonucleotide with a quenched fluor label. US 4,725,537 discloses a method for detecting the presence of a restriction cleavage site in a sample DNA molecule using two polynucleotides which are partially complementary, of which only one is labelled. However, there is no suggestion in any of these documents of a method or a polynucleotide substrate suitable for assaying for enzymes which recognise residues which do not have A-T or G-C or standard Watson-Crick base pairing.

Current methods for analysing the activity of AP endonucleases and DNA glycosylases generally use radiolabelled, or fluorescently labelled, oligonucleotides in combination with a gel electrophoresis step which is not suitable for high throughput analysis (Kreklau *et al* (2001) *Nucleic Acids Res.* 29:2558-2566; Evans *et al* (1997) *EMBO J.* 16:625-638).

The inventor has developed new assays and polynucleotides suitable for detecting the activity of DNA modifying enzymes whose recognition sites commonly feature at least one residue on the polynucleotide which does not participate in an A-T or G-C base pair which are suitable for high-

throughput detection and use in drug-screening programmes. In particular, the inventor has developed new assays and polynucleotides suitable for detecting the activity of AP endonucleases such as HAP1, DNA glycosylases and XPG-like enzymes such as XPG and XPF-ERCC1.

A first aspect of the invention provides a polynucleotide having a double stranded portion which is interrupted by at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair, the molecule further having attached thereto a fluorescent moiety and a quenching moiety which quenches the fluorescence of the fluorescent moiety.

In one embodiment, the polynucleotide is one having a double stranded portion with one strand having attached thereto a fluorescent moiety and the other strand having attached thereto a quenching moiety which quenches the fluorescence of the fluorescent moiety wherein the double stranded portion is interrupted by at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair.

A residue in a polynucleotide which does not participate in an A-T or G-C base pair is one which is not an unmodified A, G, T or C residue involved in a base-pair bond as between an unmodified adenine and thymine (A-T) or between an unmodified guanine and cytosine (G-C) base pair.

Examples of a residue which does not participate in an A-T or G-C base pair include a residue with a damaged base or which is abasic, or a residue which does not have a complementary base pairing partner on the opposite strand.

In one embodiment, a residue which does not participate in an A-T or G-C base pair is one which does not participate in standard Watson Crick base pairing. By "standard Watson-Crick" base pairing we include the meaning of the base pairing between complementary nucleotides of DNA as described in any standard molecular biology or biochemistry textbook. Hence, Watson-Crick base pairing is understood to be that which naturally occurs in DNA between two complementary bases, such as an adenine-thymine (A-T) base pair and a guanine-cytosine (G-C) base pair.

Typically, and preferably, the polynucleotide of the invention is DNA.

Typically and preferably, the polynucleotide of the invention is present in aqueous solution.

By "double stranded" we mean that the strands of the double stranded portion are associated by means of at least some A-T / G-C or standard Watson-Crick base pairing. Preferably the strands of the double stranded portion are associated in a double helix configuration. More preferably, the double stranded portion contains A-T and/or G-C base pairs and forms a regular helix, and the interruption by at least one residue which does not participate in an A-T or G-C base pair is an interruption by a portion that does not form a regular helix. Preferably, the strands are associated so as to allow quenching of the fluorescent moiety by the quenching moiety at a temperature within the range from at least about 15°C to 50°C, more preferably about 20°C to 40°C The temperature at which two associated nucleic acid strands separate depends on the number of bases in the strands, the proportion of purines and pyrimidines, the proportion of bases which are not capable of A-T or G-C or standard Watson-Crick base-pairing and the ionic strength and pH of the solution in which the polynucleotide is placed.

The number of base pairs in the polynucleotide which are associated by A-T or G-C hydrogen bondings can be any number which is sufficient to allow the two strands to associate at a temperature of at least about 20°C to 40°C (eg 37°C) or in the reaction conditions required by a mammalian DNA modifying enzyme in order for the enzyme to be able to function.

Hence, the invention provides a polynucleotide having a double stranded portion in a regular helix further having attached thereto a fluorescent moiety and a quenching moiety which quenches the fluorescence of the fluorescent moiety wherein the double stranded portion in a regular helix is interrupted by at least one residue of the polynucleotide which forms a portion which is not a regular helix. Preferably the fluorescent and quenching moieties are on different strands of the double stranded portion.

Whether a double stranded portion forms a regular helix or not can be determined by methods known in the art such as nuclear magnetic resonance and X-ray diffraction.

Preferably, the double stranded regular helix portion comprises B-form DNA and the non-helical portion produced by the at least one residue which does not form a regular helix is not B-form DNA.

It will be appreciated that, as described above, the at least one residue which does not form a regular helix can be any suitable residue including a residue with a damaged base or which is abasic, or a residue which does not have a complementary base pairing partner on the opposite strand.

The polynucleotides may be made by custom synthesis to order, for example by OSWEL Scientific (Southampton, UK) or Genset (Paris,

France). No chemical alterations or processing are necessary and the individual DNA strands are generally ready to use following synthesis.

For example, when the polynucleotide has two strands, one strand being labelled with a fluorescent moiety and the other strand being labelled with a quenching moiety, those strands may be annealed in a PCR machine (such as one provided by Omnigene, Hybaid Ltd, U.K.) for example in 500 µl batches with the fluor-labelled strand at 200 - 250 nM and the quench labelled strand at various concentrations in annealing buffer, as depicted in Figure 1, for the desired level of quenching. (Annealing buffer: 50mM Tris-HCl, pH7.5 or 8.0, 50mM NaCl, 10mM $MgCl_2$). The annealing program may consist of a 96°C melting step of 5 minutes followed by a gradual cooling to 25°C over 15 minutes. It will be appreciated that this represents a typical annealing protocol and it may readily be modified by the person skilled in the art and still achieve suitable annealing to form a polynucleotide of the invention.

Where the polynucleotide has two strands (one strand being labelled with a fluorescent moiety and the other strand being labelled with a quenching moiety) annealing of the strands can be followed by measuring the % decrease in fluorescence (i.e. the % increase in quenching) vs. added quench-labelled strand, with increasing quench-labelled strand the fluorescence falls to a minimum (Figure 1). Annealed substrates may be then optimised for excitation and emission wavelengths.

The polynucleotide may comprise a single chain or strand of residues, or may comprise two separate chains or strands of residues. By "separate" we mean not covalently bonded together. Where the polynucleotide comprises a single chain of residues, the double stranded portion may be produced by

formation of, for example, a "hairpin" type structure, where two regions of the same chain associate with each other by the chain folding back on itself. Preferably, the polynucleotide comprises two separate chains of residues.

Preferably each chain of the polynucleotide (or in the case of a polynucleotide comprising a single chain, each strand of the double stranded portion) is at least 8 or 10 residues or more in length, more preferably at least 12, 14, 16, 18, 20, 22 or 24 residues in length. The polynucleotide may be more than 40, 50, 60 or 70 or 80 residues in length. However, as will be appreciated by the skilled person, the longer the polynucleotide, the greater the cost of synthesising it and the greater the difficulty in obtaining useful quantities of pure polynucleotide. Preferably, the polynucleotide is between 25 and 35 bases in length. More preferably, the polynucleotide has sufficient length to allow binding by a DNA modifying enzyme to the polynucleotide, and to allow a useful melting temperature differential when cleaved at a cleavable bond in or adjacent to the residue which does not participate in an A-T or G-C base pair as described in more detail below.

A melting temperature differential is useful when it is sufficiently big to allow melting of at least a part of the double stranded portion polynucleotide comprising either the fluorescent moiety or quenching moiety following the action of a DNA modifying enzyme (and, in the case of a DNA glycosylase as described in more detail below, a phosphodiester bond cleavage event) on the polynucleotide at the residue which does not participate in an A-T or G-C base pair in the polynucleotide, without any change in the reaction conditions. Polynucleotides which exemplify such a feature are given below.

Preferably, when the polynucleotide comprises two separate chains, each chain has the same number of nucleotide residues although as shown in the Examples they need not have the same number of nucleotide residues. Preferably each end of the polynucleotide is "blunt-ended" such that the 5' end of each chain in the polynucleotide is A-T or G-C base-paired to the 3' end of the other chain.

The double stranded portion of the polynucleotide comprises at least two residues which form A-T or G-C base pairs, and more preferably at least 3, 4, 5, 6, 7, 8, 9 or 10 or 12 or more such residues.

Preferably all of the residues in the double stranded portion, apart from those in the interruption, are A-T or G-C base pairs.

By "interrupted" we mean that the chain of residues which are able to associate to form a double-stranded portion comprises at least one residue which does not participate in an A-T or G-C base pair. The double stranded portion may be interrupted by any number of said residues provided that association to form a double stranded portion still occurs under appropriate conditions as described above. In one embodiment, the presence of the said residue or residues may not affect the double stranded nature of the polynucleotide along its length. In another embodiment, the presence of the said residue or residues may produce a region which is not double stranded. Clearly however, if a region is produced which is not double stranded, it will be flanked on both sides by a double stranded portion.

Where the polynucleotide comprises a single chain of residues and the double stranded portion is produced by formation of, for example, a "hairpin" type structure, where two regions of the same chain associate with

each other by the chain folding back on itself, it will be understood that the residue or residues which do not participate in an A-T or G-C base pair are not those which form the loop structure of the hairpin. Rather, they interrupt the double stranded portion which is not part of the loop. This portion is often called the "stem" of the hairpin structure. When the polynucleotide of the invention has a hairpin structure it is preferred that the residue or residues which do not participate in an A-T or G-C base pair are flanked on both their 5' and 3' sides with at least one nucleotide which does form an A-T or G-C base pair. Similarly it is preferred that where there is a stretch of several contiguous residues which do not form an A-T or G-C base pair, only the 5' or 3' end of the residues at either end of that stretch (and not each individual residue within the stretch) are flanked by at least one nucleotide which does form an A-T or G-C base pair.

Typically, a polynucleotide strand comprises residues which are joined by a phosphodiester bond between each sugar and phosphate group, forming a phosphodiester backbone. Hence, a polynucleotide of the invention is one which has a phosphodiester bond joining at least two residues in the polynucleotide. Preferably, at least 10%, 20%, 50%, 70% or 90% of the covalent bonds joining the residues in the polynucleotide are phosphodiester bonds. More preferably, substantially all of the covalent bonds joining the residues in the polynucleotide are phosphodiester bonds. Where a covalent bond is not a phosphodiester bond, it may be any suitable alternative bond. Such alternatives are known in the art of synthetic polynucleotides, and include oligonucleotide mimics such as peptide nucleic acids where nucleobases are attached to a non-charged achiral polyamide backbone.

The residues in the double stranded portion of the polynucleotide may be any suitable residues which are capable of forming the double stranded

portion. Preferably, the residues are nucleic acid residues and more preferably, they are nucleotide residues. The nucleotide residues may include a ribose sugar (ie, such as is found in ribonucleic acid) or a deoxyribose sugar; preferably, the nucleotide includes a deoxyribose sugar. The nucleotide residues may include any suitable base, such as cytosine, guanine, thymine or adenine. It will be appreciated that unusual nucleotides or artificial nucleotide analogues exist which can be incorporated in the sequence of the polynucleotide and are capable of forming base pairs which do not prevent formation fo a double stranded portion or which do not affect the regular nature of a helix. An example of an unusual, naturally-occurring nucleotide is one which has inosine as the base. This base can base pair with several bases, including cytosine, adenine and uracil. When a nucleotide which has inosine as its base, pairs with adenine, the pairing is Watson-Crick.

The residue which "does not participate in an A-T or G-C base pair" can be any suitable residue, provided that it is covalently joined to the residues on either side of it typically by a phosphodiester bond. Such a residue may be an artificial residue or may be a naturally-occurring residue, such as a nucleotide residue. Where the residue is a naturally-occurring residue, the reason why it may not participate in an A-T or G-C base pair may be because it does not have an available and complementary base with which to pair. A complementary base is one which can form standard A-T or G-C bonds, as between a cytosine and guanine, and between a thymine and adenine. For example, the residue may be a cytosine base opposite a thymine base, or the opposite strand may lack any base for it to pair with, or contains a modified base which is not capable of forming an A-T or G-C base pair.

As is described in more detail below, these various types of polynucleotides are substrates for DNA glycosylases, AP endonucleases or XPG-like enzymes, which cleave at or adjacent to the residue which does not participate in an A-T or G-C base pair.

By "XPG-like" we mean an enzyme which has the phosphodiester bond cleaving activity of the enzyme XPG. This includes the enzyme XPG and the heterodimeric enzyme XPF-ERCC1.

The fluorescent moiety can be any fluorescent moiety which is capable of emitting detectable light at a given wavelength when attached to a polynucleotide, and which is capable of being quenched by a moiety which can be attached to a polynucleotide. The fluorescent moiety may be attached to the polynucleotide strand by any suitable means, but preferably it is attached to the polynucleotide covalently. Preferably, the fluorescent moiety is fluorescein. Other fluorescent moieties which are useful in the invention are any which can have their fluorescence quenched by a quenching moiety, and include those available from Molecular Probes, Inc., 4849 Pitchford Ave, Eugene, OR 97402-9165.

Convenient quenching moieties can be any moiety which reduces, preferably by at least about 50% or 70% or 80% or 90% or more, the fluorescence of the fluorescent moiety. More preferably, the quenching moiety quenches more than 99% or 99.9% or 99.99% of the fluorescence of the fluorescent moiety. Preferably, the quenching moiety quenches substantially all of the fluorescence of the fluorescent moiety when in sufficiently close proximity thereto. A molecule which is suitable for quenching fluorescein is DABCYL, available from Molecular Probes, Inc., 4849 Pitchford Ave, Eugene, OR 97402-9165. Dabcyl is a succinimidyl

ester (dabcyl, SE; D-2245) with a broad and intense visible absorption but no fluorescence, making it useful as an acceptor in fluorescence resonance energy transfer applications. The quenching moiety may also be another dye which is capable of dye-dimerisation with the fluorescent moiety leading to quenching of the fluorescence of the two dyes. Such dimerisation is known in the art, and is described in more detail in WO 99/35288. The quenching moiety may be attached to the polynucleotide by any suitable means, but preferably, the quenching moiety is covalently attached to the polynucleotide.

The polynucleotide may comprise more than one fluorescent moiety and more than one quencher moiety. However, the number of fluorescent and quencher moieties must be matched such that the polynucleotide of the invention does not fluoresce, or fluoresces to only a small extent, in the absence of a DNA modifying enzyme which is capable of using the polynucleotide as a substrate. By "fluoresces to only a small extent" we mean that the fluorescence of the fluorescent moiety when quenched by the quenching moiety is less than 50% of the maximum fluorescence in the absence of quenching by the quenching moiety. More preferably it is less than 30% or 20% or 10% or 1% or 0.1% or 0.01% of the maximum fluorescence in the absence of quenching by the quenching moiety.

Fluorescence may be measured by any suitable means, such as by exposing the fluorescence to photosensitive materials or by using a fluorimeter. Fluorimeters suitable for detecting and measuring fluorescence are well known in the art. A suitable fluorimeter which reads the fluorescence of samples in a microplate is the Spectramax Gemini available from Molecular Devices, CA, USA.

The fluorescent and quenching moieties of the polynucleotide may be located on the same strand of the double stranded portion, even where the polynucleotide comprises only a single fluorescent and quenching pair. However, the location of the fluorescent and quenching moieties must be such that the polynucleotide is a substrate for a DNA modifying enzyme such as an AP endonuclease, XPG-like enzyme or DNA glycosylase. In other words, the fluorescent and quenching moieties must not sterically obscure all of the potential cleavage sites for a DNA modying enzyme in the polynucleotide.

The fluorescent and quenching moieties of a pair (ie a quenching moiety which quenches the fluorescence of a given fluorescent moiety) may be attached on the same strand or separate strands of the double stranded portion of the polynucleotide, and may be attached to the strand or respective strands in any convenient position, configuration or orientation, provided that the relative positions of the two moieties is such that the quenching moiety can quench the fluorescence of the fluorescent moiety. The moieties may be attached to residues within the polynucleotide, as in more than 1 residue from a 5' or 3' terminus.

Where the fluorescent and quenching moieties are located on only one strand of the double stranded portion, the moieties must be attached either side of the at least one residue which does not participate in an A-T or G-C base pair. Hence, the said at least one residue will have a first moiety (a fluorescent or quenching moiety) located 5' to it and a second moiety (a quenching or fluorescent moiety respectively) whose fluorescence is quenched by, or which quenches the fluorescence of, the first moiety located 3' to it. This is required so that cleavage by a DNA modifying enzyme at or adjacent to the at least one residue which does not participate

in an A-T or G-C base pair has the potential to allow separation of the fluorescent and quenching moieties.

Preferably however, the pair or pairs of fluorescent/quencher moieties are on different strands and are attached to residues at adjacent 5' and 3' termini of the polynucleotide.

In a preferred embodiment, the polynucleotide is one which has at least one cleavable bond in one strand of the double stranded portion of the polynucleotide wherein said cleavable bond or bonds is at or adjacent to the at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair and is in a position whereby cleavage of the said cleavable bond or bonds is sufficient to dissociate at least part of the double stranded portion of the polynucleotide such that fluorescence quenching is reduced under appropriate given conditions as described above.

By "cleavable bond" we include bonds that can be broken by the action of a DNA modifying enzyme under suitable reaction conditions. Preferably the bond is one which can be cleaved by any one of an AP endonuclease, an XPG-like enzyme or a DNA glycosylase.

The nature of the reaction conditions will vary according to the enzyme used to cleave the bond. For example, most mammalian DNA modifying enzymes work optimally in the temperature range of about 20°C to about 40°C, so typically the bond should be cleavable by the enzyme within this temperature range. An example of a cleavable bond includes phosphodiester bonds which are cleaved by AP endonucleases and XPG-like enzymes, and *N*-glycosidic bonds which are cleaved by DNA glycosylases. Suitable reaction conditions with respect to, for example, pH,

ionic strength and reducing strength for any given DNA modifying enzyme are known in the art, or may be determined by routine experimentation. It will be appreciated that for the bond to be cleavable by a DNA modifying enzyme it must be in a context which indicates to the enzyme that the bond is a substrate. Hence, AP endonucleases do not cleave phosphodiester bonds indiscriminately but rather they cleave specific phosphodiester bonds which are at or adjacent to an "abasic" residue. Similarly, XPG-like enzymes cleave phosphodiester bonds which are at or adjacent to a single stranded (bubble) region of DNA which interrupts a double stranded portion, and DNA glycosylases cleave a *N*-glycosidic bond between a damaged base (or artificial analogue thereof) and a deoxyribose residues. Some DNA glycosylase enzymes, such as human Nth1 and OGG1, also possess a lysase activity which cleaves the phosphodiester backbone 3' to an AP site generated by the glycosylase activity of the enzyme.

By "quenching is reduced" we mean that the degree of quenching of fluorescence exerted by the quenching moiety in the polynucleotide following dissociation of at least part of the double stranded portion is less than it was before dissociation occurred, and this decrease is a detectable decrease. Preferably, the degree of quenching is reduced by at least about 10%, 30% or 50%. More preferably, the quenching is reduced by more than about 70%, 90% or 95%. Preferably, substantially all of the quenching is eliminated. It will be appreciated that a reduction in quenching is typically measured by an increase in fluorescence of the fluorescent moiety.

Typically, the polynucleotide is a substrate for any one of the DNA modifying enzymes including AP endonucleases such as HAP1, Nth1 or OGG1, XPG-like enzymes such as XPG or XPF-ERCC1, or a DNA glycosylase such as Nth1, OGG1 or AAG. AAG is an abbreviation for

alkyladenine glycosylase, although the enzyme is also known as 3-methyladenine DNA glycosylase, MPG or ANPG or may also be called 3AAG.

The polynucleotides of the present invention include four distinct types of substrate (which, for convenience, we shall call types I, II, III and IV).

Polynucleotides as substrates for AP endonucleases (Type I substrates)

According to a preferred embodiment, the at least one residue in the polynucleotide which does not participate in an A-T or G-C base pair is an abasic residue and the polynucleotide is a substrate for an AP endonuclease. Abasic residues are known in the art, and are residues which do not have any purine or pyrimidine base, such as any one of cytosine, adenine, thymine or guanine, attached to the sugar residue. Such abasic residues can be naturally occurring, or may be artificial. An example of an artificial abasic residue is a residue which has tetrahydrofuran in place of a base. Other examples of artificial abasic residues are described in Pompizi *et al* (2000) *Nucleic Acids Res.* 28:2702-2708.

Hence, a polynucleotide of this embodiment includes a polynucleotide with at least one abasic residue within a double stranded portion, which polynucleotide comprises a fluorescent moiety attached to one strand of the double stranded portion and a quenching moiety attached to the other strand which is located such that it is able to quench the fluorescence of the fluorescent moiety. The at least one abasic residue is located within the polynucleotide such that cleavage of the phosphodiester bond at or adjacent to said residue or residues allows a fragment of the polynucleotide to melt from the rest of the polynucleotide under appropriate conditions as

discussed above. This fragmentation of the polynucleotide separates the fluorescent moiety and the quenching moiety, thereby reducing quenching of the fluorescent moiety by the quenching moiety. This leads to a detectable increase in fluorescence.

Preferably, the fragment which can be produced and released from the polynucleotide by cleavage of the phosphodiester backbone by the AP endonuclease comprises a 5' or 3' terminus of the polynucleotide.

In order for quenching of the fluorescent moiety by the quenching moiety to be reduced following cleavage, the fragment of the polynucleotide which is able to melt from the polynucleotide will comprise either the fluorescent or quenching moiety of the polynucleotide. Preferably, the fluorescent and quenching moieties in the polynucleotide are located at the 5' and adjacent 3' terminus, respectively, of the original polynucleotide, and a fragment comprising one of these 5' or 3' termini (ie having the fluorescent moiety or quenching moiety attached thereto) is released following cleavage by the AP endonuclease. Clearly, in an alternative embodiment, the quenching moiety may be located at the 5' end and the fluorescent moiety may be located at the 3' end.

More preferably, the phosphodiester bond which is cleaved by an AP endonuclease is located within no more than 10, 9, 8, 7, 6, 5, 4, 3 or 2 or 1 residues of the 5' or 3' terminus of the strand of the original polynucleotide which contains the at least one abasic residue and which is labelled with the fluorescent or quenching moiety. More preferably, the bond is located 6 or 7 residues from the adjacent 3' and 5' termini which have the fluorescent and quenching moieties attached thereto. An advantage of this location is that it allows a fragment comprising either the fluorescent or quenching

moiety to melt from the polynucleotide under typical reaction conditions, with separation of the fluorescent and quenching moieties and reduction of quenching of the fluorescent moiety.

An example of a polynucleotide according to this embodiment is shown below (type I):

```
5′   fluor -GCCCCCXGGGGACGTACGATATCCCGCTCC-3′
            :::::: ::::::::::::::::::::::::
3′   quench-CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5′
```

wherein ":" indicates A-T or G-C base pairing which produces a double stranded portion, "X" indicates an abasic residue which does not form A-T or G-C base pairs and which interrupts the double stranded portion, "fluor" represents a fluorescent moiety which is being quenched by "quench", a quenching moiety. The fluorescent and quenching moieties are attached to adjacent 5' and 3' termini of different strands.

In this embodiment there is a single abasic residue on the same strand to which the fluorescent moiety is attached. In other embodiments, the quenching moiety may be attached to this strand and the fluorescent moiety to the other strand.

Clearly, under appropriate conditions, cleavage of the phosphodiester backbone at the position of or adjacent to the abasic residue (X) would permit the 5' fluorescent-labelled fragment to melt from the rest of the polynucleotide. This melting would separate the fluorescent and quenching moieties, and reduce the quenching of the fluorescent moiety:

```
5′   *fluor -GCCCCC

                    and

                        XGGGGACGTACGATATCCCGCTCC-3′
                        :::::::::::::::::::::::::
3′   quench-CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5′
```

As before, ":" indicates A-T or G-C base pairing, but "*fluor" indicates a fluorescent moiety attached to the melted fragment, which has reduced quenching by the quenching moiety (quench).

It will be appreciated that the polynucleotide shown above is merely one specific embodiment, and the specific polynucleotide may be changed according to the practice of the invention. In particular, the G-C and A-T contents of the double stranded portions of the polynucleotide may be varied so as to modify the temperature of dissociation, and the number of base pairs in the polynucleotide. As is well known, G-C base pairs are more stable than A-T base pairs because of having three rather than two hydrogen bonds. This can be taken into account when designing and making other embodiments of the invention. For example, if A-T base pairs are to be incorporated in the double stranded portion between the abasic residue (X) and the fluorescent moiety (fluor) then more of them are required than G-C base pairs to maintain the same melting temperature of the polynucleotide.

Polynucleotides as substrates for XPG-like enzymes (Type II substrates)

In an alternative preferred embodiment, the at least one residue which does

not participate in an A-T or G-C base pair is a residue without a complementary partner in the other strand or does not base pair with its partner in the other strand and hence the polynucleotide is a substrate for an XPG-like enzyme. Hence, in this embodiment the polynucleotide contains residues in a strand which have no A-T or G-C base pairing with a residue on the other strand of the double stranded portion because, for example, they are non-complementary with that residue. The polynucleotide may have only one such non-complementary residue, or may have more than one said residue. Preferably, the polynucleotide has more than two or three. More preferably, the polynucleotide has more than five said residues and less than 70, 60 or 50 . Thus, suitable polynucleotides are those with "bubbles" which interrupt the double-stranded portion and each strand of the bubble may independently have, for example, 10 or 15 or 20 or 25 or 30 or 35 or 40 residues. Where the polynucleotide has more than one non-complementary residue, it is preferred if the said residues occur consecutively on the same strand. Of course, there will be non-complementary residues on the other strand. Suitable bubbles of residues which do not participate in an A-T or G-C base pair are described in Evans *et al* (1997) *EMBO J.* 16:625-638.

It is known that eukaryotic DNA glycosylases may also recognise single base mismatches within a polynucleotide as a substrate (Krokan *et al* (1997) *Biochem. J.* 1-16). Hence, in one embodiment, the polynucleotide has only one non-complementary residue which interrupts the double stranded portion of the type II substrate (ie, it is a single base mismatch, where a residue on one strand does not have a complementary partner on the opposite strand with which to form an A-T or G-C base pair.

Preferably, where the polynucleotide is to be a substrate for an enzyme involved in nucleotide excision repair (NER), such as XPG-like enzymes, the polynucleotide has more than four consecutive non-complementary residues.

Hence, a polynucleotide of this embodiment includes a polynucleotide with a double stranded portion with a fluorescent moiety attached to one strand and a quenching moiety attached to the other strand which is located such that it is able to quench the fluorescence of the fluorescent moiety wherein the double stranded portion is interrupted by at least one residue which does not have a complementary base on the other strand. The at least one said residue (preferably at least 5 consecutive said residues) is located within the polynucleotide such that cleavage of a phosphodiester bond at or adjacent to said residue (preferably 5' to the said residue) reduces quenching of the fluorescence of the fluorescent moiety by the quenching moiety.

It is preferred if the phosphodiester bond which is cleaved by an XPG-like enzyme (such as XPG or XPF-ECCR1) is located within no more than 10, 9, 8, 7, 6, 5, 4, 3 or 2 or 1 residues of the 5' or 3' terminus of the original polynucleotide which is labelled with the fluorescent or quenching moiety. More preferably, the bond is located 6 or 7 residues from the 5' or 3' terminus.

An example of a polynucleotide according to this embodiment is shown below (type II):

```
                    CCCCCCCCCCCCCCC
5' fluor-CGCTC                  AGCGCCACGTTGCTGCCC-3'
```

```
          :::::                    ::::::::::::::::::
3'quench-GCGAG                     TCGCGGTGCAACGACGGG-5'
                 TTTTTTTTTTTTTTT
```

As explained above, "fluor" represents a fluorescent moiety which is being quenched by "quench", a quenching moiety. In this case, the residues interrupting the double stranded portion are non-complementary residues which do not base pair, indicated in italics and by the increased distance between the bases, forming a "bubble". This section of non-complementary bases represents a recognition sequence for the enzymes XPG and XPF-ERCC1. In the case of XPG, which cleave 5' to the end of the non-base paired section, either of two possible products can be produced:

```
                 CCCCCCCCCCCCCCC
5'*fluor-CGCTC
```

and

```
                                   AGCGCCACGTTGCTGCCC-3'
                                   ::::::::::::::::::
3'quench-GCGAG                     TCGCGGTGCAACGACGGG-5'
                 TTTTTTTTTTTTTTT
```

Or potentially

```
                 CCCCCCCCCCCCCCC
5'*fluor-CGCTC                     AGCGCCACGTTGCTGCCC-3'
                                   ::::::::::::::::::
                                   TCGCGGTGCAACGACGGG-5'
                 TTTTTTTTTTTTTTT
```

and

3′quench-GCGAG

It will be appreciated that the polynucleotide shown above is merely one specific embodiment and the specific polynucleotide may vary according to the practice of the invention. As discussed above in relation to the AP endonuclease substrates, the G-C and A-T contents of the doubled stranded portions of the polynucleotides may be varied so as to modify the temperature of dissociation and the number of base pairs in the polynucleotide.

Although the non-complementary bases in this example are A and C, other non-complementary pairs may be used such as G and A, T and C, and T and G. Also, although in this example the same nucleotides (As) form one strand of the "bubble" and the same non-complementary nucleotides (Cs) form the other strand of the bubble, it is possible that different types of nucleotides could be used in each strand so long as non-complementarity is maintained. In addition, although in this example each strand of the "bubble" has twelve nucleotide residues, the number can vary, preferably between about 5 and 70. Preferably, the non-complementary nucleotides within the "bubble" are not complementary to another region of the polynucleotide, so as to avoid undesirable or alternative annealing during preparation of the polynucleotide.

Polynucleotides as substrates for DNA glycosylases (Types III and IV)

According to a further preferred embodiment, the at least one residue which does not participate in an A-T or G-C base pair has a damaged base. Damaged bases are those bases which contain chemical groups not found in the normal DNA bases and so, according to the invention, are ones which are recognised by DNA glycosylases as being incorrectly included in DNA. Such bases include alkylated, halogenated, oxidised, ring-fragmented and deaminated bases, and include 3-methyladenine, 5,6-dihydrouracil, 5-hydroxy-5-methylhydantoin, 8-oxoguanine and 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (formamidopyrimidine). The bases inosine and uracil are considered damaged bases in the context of this embodiment of the invention since they are recognised by certain DNA glycosylases as not normal DNA bases as shown in the Examples. Typically, damaged bases include those damaged bases described in Krokan *et al* (1997) *Biochem. J.* 325:1-16. Also typically, polynucleotides comprising damaged bases are substrates for DNA glycosylase enzymes such as Nth1, 3AAG and OGG1.

According to this embodiment, the polynucleotide may contain only one residue with a damaged base, or may contain several such residues. Where the polynucleotide contains several such residues, the said residues may be located all on the same strand, or may be present on both strands. Furthermore, the residues may be adjacent or may be interspersed throughout the double stranded portion.

Two types of polynucleotides of the invention (which we will call types III and IV polynucleotides) are suitable as substrates for DNA glycosylases. However, they are distinct in the number and/or location of the residue(s) with a damaged base, and hence different products may be generated following the action of a DNA glycosylase on the polynucleotide.

Type III substrate

A first polynucleotide of this embodiment includes a polynucleotide where cleavage of an *N*-glycosidic bond in the at least one residue with a damaged base produces an abasic residue which does not decrease the melting temperature of the polynucleotide to allow at least a portion of the polynucleotide to melt in a given set of conditions. However, cleavage of the resultant polynucleotide at the phosphodiester bond at or adjacent to the abasic residue or residues (created upon action by the DNA glycosylase) does allow melting of at least a portion of the double stranded portion of the polynucleotide to reduce quenching of the fluorescence of the fluorescent moiety by the quenching moiety.

Hence the type III polynucleotide is one with a double stranded portion which has a fluorescent moiety attached to one strand and a quenching moiety attached to the other strand which is located such that it is able to quench the fluorescence of the fluorescent moiety wherein the double stranded portion is interrupted by at least one residue which has a damaged base whereby conversion of said residue or residues into abasic residues and subsequent cleavage of the phosphodiester bond at or adjacent to the abasic residues produces a decrease in quenching of the fluorescence of the fluorescent moiety by the quenching moiety.

The polynucleotide may have any number of residues with a damaged base. Conveniently, where the polynucleotide according to this embodiment has only one residue with a damaged base, the said base is located within less than ten bases of a 5' terminus (or 3' terminus), and the fluor and quencher moieties are located at, or in the less than ten bases from, the same 5' terminus (or 3' terminus as the case may be) (see below).

It is preferred if the residue with a damaged base is located within no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 residues of the 5' or 3' terminus of the original polynucleotide which is labelled with the fluorescent or quenching moiety. More preferably, the bond is located 6 or 7 residues from the 5' or 3' terminus.

An example of such a polynucleotide is shown below (type III):

```
5' fluor-     GCCCCCOGGGGACGTACGATATCCCGCTCC-3'
              ::::::;::::::::::::::::::::::
3' quench-    CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5'
```

whereby O indicates a residue with a damaged base such as inosine or uracil, ":" indicates A-T or G-C base pairing and ";" indicates non-A-T or G-C base pairing.

Incubation of this polynucleotide with a DNA glycosylase would produce the following polynucleotide:

```
5'fluor- GCCCCCXGGGGACGTACGATATCCCGCTCC-3'
         :::::: ::::::::::::::::::::::::
3'quench-CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5'
```

wherein "X" indicates an abasic residue. Cleavage of the phosphodiester bond at or adjacent to the abasic residue results in the following fragment and polynucleotide:

```
5'*fluor-GCCCCC
```

and

```
                    XGGGGACGTACGATATCCCGCTCC-3'
                    :::::::::::::::::::::::::
3'quench-CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5'
```

wherein "*fluor" indicates a fluorescent moiety with reduced quenching by the quenching moiety "quench".

Cleavage of the phosphodiester bond at or adjacent to the abasic residue may be achieved by any of several possible means, including by any AP lyase activity of the DNA glycosylase (both Nth1 and OGG1 have intrinsic AP lyase activity), using an AP endonuclease such as HAP1 as described above or may be achieved using chemical means, for example using chemical cleavage by a diamine such as *N,N'*-dimethylethylenediamine as described in McHugh and Knowland (1995) *Nucleic Acids Res.* 23:1664-1670 or by other molecules as described in Constant *et al* (1990) *Anti-Cancer Drug Design* 5:59-62.

As described above in relation to the AP endonuclease substrates, it is preferred if the residue which is recognised by DNA glycosylase is located within no more than 10, 9, 8, 7, 6, 5, 4, 3 or 2 or 1 residues of the 5' or 3' terminus of the strand of the original polynucleotide which contains the residue recognised by DNA glycosylase and which is labelled with the fluorescent or quenching moiety. More preferably, the bond is located 6 or 7 residues from the 5' or 3' terminus.

Type IV substrate

A second polynucleotide of this embodiment includes a polynucleotide with a double stranded portion which has a fluorescent moiety attached to one strand and a quenching moiety attached to the other strand which is located such that it is able to quench the fluorescence of the fluorescent moiety wherein the double stranded portion is interrupted by at least one residue which has a damaged base wherein the proportion and/or distribution of said residue or residues in the polynucleotide is such that conversion of at least one of the said residue or residues to an abasic residue or residues reduces the melting temperature of the polynucleotide such that quenching of the fluorescent moiety is reduced.

It will be appreciated that since the polynucleotide of this embodiment includes one where cleavage of at least one of the cleavable bond or bonds present within such residues into abasic residues is sufficient to dissociate or melt at least part of the double stranded portion of the polynucleotide such that fluorescence quenching is reduced, the number of residues with a damaged base which are required may vary according to the length of the polynucleotide, the location of the fluorescent moieties, the proportion of G-C and A-T pairs for given reaction conditions. Typically, when there is more than one residue with a damaged base they are on the same strand of the double stranded portion.

An example of such a polynucleotide is shown below (type IV):

```
5'fluor- GCCCCCCTATCCCGCTCCTAT-3'
         :;:::;:::::::::;::::
3'quench-COGGOGOATAGGGOGAOGATA-5'
```

wherein the "O" indicates a residue with a damaged base such as inosine or uracil, ";" indicates non-A-T or G-C base pairing and ":" indicates A-T or G-C base pairing. "quench" indicates a quenching moiety which decreases the fluorescence of the fluorescent moiety "fluor".

Incubation of such a polynucleotide with a DNA glycosylase would produce the product:

5'*fluor-GCCCCCCTATCCCGCTCCTAT-3'

and

3'quench-CXGGXGXATAGGGXGAXGATA-5'

wherein "X" indicates an abasic residue, and "*fluor" indicates a fluorescent moiety with reduced quenching by the quenching moiety.

As described above in respect to the type I and type II polynucleotides, it will be appreciated that the type III and type IV polynucleotides shown above are merely specific embodiments, and the specific polynucleotide may be changed according to the practice of the invention. In particular, the proportion of G-C and A-T base pairs may be varied to alter the melting properties of the polynucleotide, and the location of the residues which have a damaged base may be altered.

Strands of nucleic acid of a chosen sequence may be made by any means known in the art of oligonucleotide synthesis, and said strands can be annealed to form a duplex polynucleotide with at least one portion of complementary base-pairing by standard laboratory methods.

As described above, it will be appreciated that the double stranded portion of the polynucleotide of the invention is preferably one which contains A-T and/or G-C base pairs and forms a regular helix, and that the interruption by at least one residue which does not participate in an A-T or G-C base pair is an interruption by a portion that does not form a regular helix.

A second aspect of the invention provides a method of assaying for the activity of a DNA modifying enzyme, the method comprising

(i) incubating the enzyme with a polynucleotide according to the first aspect of the invention; and

(ii) detecting fluorescence.

Conveniently, the assay is carried out in suitable reaction conditions, preferably those that are optimal for the DNA modifying enzyme in question in terms of temperature, pH, ionic strength. The suitable conditions for the enzymes HAP1 and XPG include those described in the Examples. Suitable conditions for the DNA glycosylase 3AAG include 25mM HEPES-KOH, 0.5mM DTT, 0.5mM EDTA, 150mM KCl, 1% glycerol, pH 7.8, whereas Nth1 may be assayed in 66 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. Other suitable conditions for AAG are given in the Examples below.

It will be appreciated that other suitable conditions are known in the art or can be arrived at by the skilled person by routine experimentation without undue effort.

The enzyme may be any suitable DNA modifying enzyme which is capable of cleaving a bond in the polynucleotide substrate. Preferably, the enzyme is any one of an AP endonuclease such HAP1, Nth1 or OGG1, or an XPG-like enzyme such as XPG and XPF-ERCC1 or a DNA glycosylase such as 3AAG, Nth1 or OGG1. The DNA sequences coding for these enzymes are known; the sequence of HAP1 is given under GenBank Accession No XM_007490, XPG is given under GenBank Accession No XM_007128, 3AAG is given under GenBank Accession No XM_008125 and the encoding sequence of human Nth1 is given under GenBank Accession No XM_007930. The enzymes may be produced by recombinant DNA means using methods well known in the art. At least some of the enzymes are commercially available (eg HAP1 is available from Trevigen Ltd. Gaithersburg, MD, USA)

As discussed above, fluorescence detection can be done by any convenient means, such as by using a fluorimeter. Detection may be simply the determination of the presence of fluorescence, or may be the measurement of the extent of fluorescence. Preferably, the extent of fluorescence is measured.

It will be appreciated from the foregoing disclosure of the polynucleotides of the first aspect of the invention that they are useful as substrates for certain DNA modifying enzymes as described and that action of the enzyme on the substrate leads directly (or indirectly in the case of one particular DNA glycosylase assay) to cleavage of a bond of the polynucleotide which leads to destabilisation of the polynucleotide under reaction conditions such that the portion of the polynucleotide containing the fluorescent moiety and the portion of the polynucleotide containing the quenching moiety dissociate leading to an increase in fluorescence (decrease in quenching).

The assay has different embodiments which may be grouped according to the nature of the substrate used and the enzyme being assayed. These embodiments are discussed in more detail below.

However, in each assay the differential melting points of an intact polynucleotide of the first aspect of the invention (substrate) compared to a polynucleotide which has been cleaved by a DNA modifying enzyme is exploited to allow the quench and fluor to dissociate and give rise to a positive signal. Thus, in the intact polynucleotide substrate the quenching moiety and fluorescent moiety are juxtaposed so that quenching occurs but upon cleavage with the enzyme the two moieties are no longer juxtaposed and an increase in fluorescence occurs.

The intact polynucleotide (substrate) may have some fluorescence in the absence of enzyme and this may be measured as a baseline before the addition of the enzyme to be assayed.

Action of the DNA modifying enzyme on the substrate (a polynucleotide of the invention) will produce at least two products which each have a single stranded portion and which each comprise either a fluorescent moiety or a quenching moiety. Advantageously, fluorescence by the product containing the fluorescent moiety may be promoted by addition of an excess of single stranded, non-labelled nucleic acid. This single-stranded, non-labelled nucleic acid must be complementary in sequence to at least part (eg, at least 3, 4, 5, 6, 7, 8, 9, or 10 or 12 or 15 or more residues), of the single-stranded portion of either the single stranded product comprising a fluorescent moiety or the single-stranded product comprising a quenching moiety. By an "excess" we mean at least 1 fold, 10 fold, 100 fold excess over the

number of labelled molecules of substrate. Preferably, the number of single stranded unlabelled molecules added is more than 1000 fold the number of labelled molecules of substrate.

Although it may be sufficient to detect or measure the fluorescent emission only once following step (i), for example, 50 seconds, 100, 200, 300, 400, 700, 900 seconds (15 minutes) or 30 minutes after combining the enzyme with the polynucleotide (ie discontinuous assay), it may be useful to measure the fluorescence at more than one time point following the combination to determine the kinetics of the enzyme activity (ie continuous assay). The most convenient time point(s) for detecting or measuring fluorescence may vary according to the particular enzyme being assayed, and the temperature at which the assay is conducted.

The method may be conducted at any suitable temperature at which the enzyme is active and at which the polynucleotide has at least a portion in a double stranded form at the point at which it is added to the enzyme.

Assays using type I substrates (eg assays of AP endonucleases)

In this embodiment, the enzyme being assayed is an enzyme with AP endonuclease activity (ie, an AP endonuclease), such as HAP1. It is preferred if the polynucleotide is a type I polynucleotide as described above. Hence, the polynucleotide is a polynucleotide comprising a double stranded portion with one strand having a fluorescent moiety attached and the other strand having attached a quenching moiety which quenches the fluorescence of the fluorescent moiety, wherein the double stranded portion is interrupted by at least one abasic residue. The at least one abasic residue is located

within the polynucleotide such that cleavage of the phosphodiester bond at or adjacent to said residue reduces quenching of the fluorescent moiety.

The reduction of quenching of the fluorescent moiety is achieved by the melting of a fragment containing either the quenching or fluorescent moiety from the rest of the polynucleotide following cleavage of the phosphodiester bond at or adjacent to the abasic residue, and this can be measured by detecting the increase in fluorescence of the polynucleotide.

As described above, the polynucleotide may have initial fluorescence before any cleavage of the phosphodiester backbone. Hence, activity of the enzyme may be determined by comparing the initial, baseline of fluorescence with that produced following incubation of the polynucleotide with the enzyme being assayed. An increase in fluorescence indicates enzyme activity.

This embodiment of the invention provides a method of assaying for the activity of an AP endonuclease the method comprising the steps of

(i) incubating the AP endonuclease with a type I polynucleotide substrate; and

(ii) detecting fluorescence.

Whilst simply detecting an increase in fluorescence is indicative of activity of the enzyme, determining the rate of increase in fluorescence may indicate the rate of the enzyme activity. Information regarding the rate of enzyme activity may be useful in methods of identifying inhibitors of the enzyme activity, as described in more detail below.

The fluorescence may be detected from the moment that the AP endonuclease enzyme and polynucleotide are combined. Alternatively, the fluorescence may be detected following a time interval, or a change in the reaction conditions. For example, it may be useful to increase the temperature of the reaction to promote optimal melting of the fragment produced by the enzyme, or to inactivate the enzyme and halt its activity. It will be appreciated that where the temperature is increased for any reason, it should not be increased to the extent that the polynucleotide melts regardless of the absence of any enzyme activity, or in addition to the melting caused by any enzyme activity. Such a temperature increase could separate the fluorescent and quencher moieties and produce a decrease in the quenching of the fluorescent moiety, which would cause an increase in fluorescence without necessarily indicating any enzyme activity.

Preferably, the type I substrate used in this embodiment is one where the abasic residue is located less than 10 nucleotides from a 5' or 3' terminus which is attached to a fluorescent or quenching moiety. More preferably, the abasic residue is located at 7 residues or 6 residues or 5 residues from the 5' or 3' terminus. An advantage of this position of the abasic residue is that melting of the fragment produced is more likely to occur under the enzyme reaction conditions immediately following cleavage of the phosphodiester bond by the AP endonuclease without any change in the reaction conditions (eg increase in temperature). This allows for real-time, continuous monitoring of enzyme activity.

<u>Assays using type II substrates (eg assays for XPG-like enzymes)</u>

In another embodiment, the enzyme whose activity is being assayed is an XPG-type enzyme, such as XPG or XPF-ERCC1. In this embodiment, it is preferred if the polynucleotide is a type II substrate as defined above. Hence, a suitable polynucleotide for use in this embodiment is one which comprises a double stranded portion with one strand having a fluorescent moiety attached and the other strand having attached a quenching moiety which quenches the fluorescence of the fluorescent moiety, wherein the doubled stranded portion is interrupted by at least one residue which does not have a complementary base on the other strand of the double stranded portion. The least one said residue is located within the polynucleotide such that cleavage of a phosphodiester bond at or adjacent to said residue reduces quenching of the fluorescent moiety.

This embodiment of the invention provides a method of assaying for the activity of an XPG-like enzyme the method comprising the steps of

(i) incubating the XPG-like enzyme with a type II polynucleotide substrate; and

(ii) detecting fluorescence.

As described above, the type II polynucleotide may have any number of non-complementary residues. Preferably, the polynucleotide has at least two, three, four, five or six consecutive such residues so that a section of non-complementarity which is a substrate of an XPG-like enzyme is produced.

As described above in relation to assays using type I substrates, the reaction conditions may be varied prior to detecting the fluorescence, and the fluorescence may be detected continuously or discontinuously.

Also as described above, it is preferable if the at least one non-complementary residue is located less than 10 nucleotides from a 5' or 3' terminus which is attached to a fluorescent or quenching moiety. More preferably, the abasic residue is located at 7 residues or 6 residues or 5 residues from the 5' or 3' terminus.

Assays using type III substrates (eg assays for DNA glycosylases)

In a further embodiment, the activity of the enzyme being assayed is a conversion of a residue with a damaged base to an abasic residue. In this case the enzyme is a DNA glycosylase, and preferably the enzyme is 3AAG, Nth1 or OGG1.

Typically, the residue which has a damaged base, is one such as 8-oxoguanine or formamidopyrimidine as described above. It is preferred that the polynucleotide used in this embodiment is a type III substrate as defined above.

Hence, it will be appreciated that polynucleotides of the invention which are useful in this embodiment are those comprising a double stranded portion with one strand having a fluorescent moiety attached and the other strand having attached a quenching moiety which quenches the fluorescence of the fluorescent moiety, wherein the doubled stranded portion is interrupted by at least one residue which has a damaged base whereby conversion of the said residue or residues into abasic residues and subsequent cleavage of the phosphodiester bond at or adjacent to the abasic residue produces a decrease in quenching of the fluorescence or adjacent to the fluorescent moiety by the quenching moiety.

According to this embodiment, the assay preferably further comprises the step of cleaving the polynucleotide at any abasic residues before detecting the fluorescence. Hence, this embodiment provides a method of assaying for the activity of a DNA glycosylase, the method comprising

(i) incubating the DNA glycosylase with a type III polynucleotide substrate;

(ii) cleaving the polynucleotide produced from step (i) at or adjacent to any abasic residues; and

(iii) detecting fluorescence.

The cleavage may be achieved by any means which is capable of selectively cleaving the phosphodiester backbone at or adjacent to the residue which has been converted to an abasic residue. Where the DNA glycosylase also has AP lyase activity (such as is the case with Nth1 and OGG1), the cleavage may be achieved by the DNA glycosylase being assayed. Alternatively, cleavage may be achieved by addition of a further component. Suitable means include the addition of an AP endonuclease such as HAP1, which selectively cleaves at or adjacent to abasic residues, and the use of chemical reagents such as diamines which are described in McHugh and Knowland (1995) *Nucleic Acids Res.* 23:1664-1670 or by other molecules as described in Constant *et al* (1990) *Anti-Cancer Drug Design* 5:59-62. As is shown in the Examples, steps (i) and (ii) may occur in the same reaction for example when diamine is present in the glycosylase reaction mixture.

It will be appreciated that it may be necessary to change the reaction conditions in order to achieve cleavage at the abasic site.

It will be appreciated that the product of step (i) is, effectively, a type I polynucleotide as defined above, and steps (ii) and (iii) may be steps (i) and (ii) of an AP endonuclease assay as described above. Thus, one embodiment of the DNA glycosylase of the invention is a coupled assay wherein the product of the action of DNA glycosylase on the substrate is the substrate for an AP endonuclease such as HAP1.

Where the enzyme used in this embodiment (and a further embodiment described below) is Nth1, then the assay may usefully comprise the further step of including the enzyme XPG in the incubation components of step (i). The XPG may be added to the components in the incubation of step (i) at any stage of the preparation of the incubation, but it is preferred if it is added prior to addition of the polynucleotide; ie, before the Nth1 is exposed to its substrate. XPG is believed to serve as a cofactor for the efficient function of Nth1 and promote binding of Nth1 to damaged DNA (Klungland *et al* (1999) *Mol. Cell* 3:33-42). The XPG may be present as an active, wild type enzyme, or may be provided as a catalytic site mutant which is itself inactive in nucleotide excision repair. A suitable catalytic site mutant of XPG is described in Klungland *et al* (1999) *Mol. Cell* 3:33-42. Where XPG is included in the incubation of step (i), it is preferred that it is present as an inactive catalytic site mutant

Similarly, the Y Box binding protein 1 (YB-1; also identified as DNA binding protein B (DbpB)) is also considered to be a stimulator of Nth1 (Marenstein *et al* (2001) *J. Biol. Chem.* 276;21242-9), and for this reason, where the DNA modifying enzyme in the assay is Nth1, it may be useful or beneficial to include YB-1 in the incubation of step (i). It will be appreciated that either or both YB-1 and XPG may be added to Nth1 in the incubation of step (i) in order to stimulate the activity of Nth1.

Assays using type IV substrates (eg assays for DNA glycosylases)

A still further embodiment provides another means of assaying for the activity of a DNA glycosylase. In this embodiment, it is preferred if the polynucleotide is a type IV polynucleotide. Hence, it will be appreciated that polynucleotides of the invention which are useful in this embodiment are those wherein a double stranded portion is interrupted by at least one residue which has a damaged base wherein the at least one said residue is located within the polynucleotide such that cleavage of an *N*-glycosidic bond in the residue produces an abasic residue. Typically, in the polynucleotide the proportion or distribution of residues which have an oxidative base lesion is such to permit melting of the two strands of the polynucleotide upon their conversion to abasic residues. In other words, it is preferred if the polynucleotide has more than one residue with a damaged base, preferably at least 2, 3, 4, 5, 6 or 7 or more. It will be appreciated that the number of said residues required to produce the desired melting properties may be determined by the characteristics of the double stranded portion of the polynucleotide, such as its length and G-C or A-T content.

Clearly, it is preferred if the residues with a damaged base (type IV) are distributed throughout the doubled stranded portion in such a way that the action of the DNA glycosylase allows melting of the at least the portion to which the fluorescent moiety or quenching moiety is attached. Hence, it is preferred, but not essential for the entire double stranded portion to melt following cleavage of *N*-glycosidic bonds by the DNA glycosylase, provided that the portion which does melt allows the separation of the

fluorescent and quenching moieties so that quenching of the fluorescent moiety by the quenching moiety is detectably reduced.

The melting of the two strands in the double stranded portion of the polynucleotide may be produced solely by the decrease in the melting temperature caused by the loss of hydrogen bonding from the residue which has a damaged base when it is converted to an abasic residue, without any change in the reaction conditions. Alternatively, the melting of the strands may be produced by a decrease in the melting temperature coupled with a change in the reaction conditions. Such a change may be an increase in temperature or a decrease in ionic strength.

As described above in relation to assays which use a type III substrate, where the enzyme used in this embodiment is Nth1, then the assay may usefully comprise the further step of including the enzyme XPG in the incubation components of step (i). Where XPG is included in the incubation of step (i), it is preferred that it is present as an inactive catalytic site mutant.

Hence, this embodiment of the invention also provides a method of assaying for the activity of a DNA glycosylase using a type IV substrate, the method comprising

(i) incubating the DNA glycosylase with a type IV polynucleotide substrate and the enzyme XPG or an inactive mutant thereof; and

(ii) detecting fluorescence.

Clearly, the assay of this embodiment does not require any cleavage of the phosphodiester backbone by, for example, HAP1, in order to reduce

quenching of the fluorescence of the fluorescent moiety in the polynucleotide. However, the assay may still beneficially include the further step of adding the enzyme HAP1 after the incubation of step (i) but prior to detection of the fluorescence and prior to any chemical cleavage step. This is because it is believed that the enzyme HAP1 may promote the dissociation of the DNA glycosylase from the polynucleotide substrate, thereby optimising any separation of DNA strands and consequently optimising any reduction in quenching of fluorescence. Where HAP1 is included for this reason, catalytically inactive HAP1 may be used, although this does not stimulate glycosylase activity to the same extent that active HAP1 can (Hill *et al* (2001) *Nucleic Acids Res.* 29:430-438).

It is known that in cancer cells, the level of a DNA modifying enzyme such as XPF-ERCC1 may be low (Köberle *et al* (1999) *Current Biology* 9:273-276), or that a defect in cellular production of a DNA modifying enzyme may promote cancer (Vilpo *et al* (1995) *Mol. Cell. Biol.* 15:290-297). Low levels of such an enzyme may be indicative of a disease state such as cancer, or of a sensitivity to a particular treatment, such as platinum (eg cisplatin) based therapy, or of the likelihood of developing cancer. It is also reported that elevated expression of HAP1 in NT2 cells confers resistance to both bleomycin and radiation (see Robertson, K. A. *et al.* (2001) *Cancer Res.* 61, 2220-2225). It is also known that nuclear expression of HAP1 in head-and-neck cancer is associated with resistance to chemoradiotherapy (see Koukourakis, M. D. *et al.* (2001) *Int. J. Radiation Oncology Biol. Phys.* 50(1), 27-36).

Hence, it may be useful to determine the level of a DNA modifying enzyme in a patient to diagnose or prognose the disease or likely response to a particular therapy. The assays of the present invention may be useful in

indicating the level of enzyme in a cell in terms of its activity, thereby allowing a diagnosis or prognosis to be made.

Where the assay of the invention is to be used to prognose or diagnose cancer in a patient, or determine the sensitivity of a patient to a cancer therapy such as radiation or a platinum based therapeutic, then the enzyme is typically provided in a sample containing cells from the patient. More preferably, those cells from the patient are ones in which cancer has been found, or is suspected.

Conveniently, the enzyme whose activity is being assayed is an AP endonuclease such as HAP1 or an XPG-like enzyme such as XPF-ERCC1.

Therefore, a further embodiment of the invention provides a method for diagnosing and/or prognosing cancer in a patient comprising the steps of

(i) incubating a sample containing cells from the patient with a polynucleotide according to the first aspect of the invention;
(ii) detecting fluorescence; and
(iii) comparing the fluorescence of step (ii) with that produced by a control sample.

A "control sample" is one in which cancer is not found or suspected. Preferably, the control sample is from the patient.

Where the polynucleotide substrate being used is a substrate for an XPG-like enzyme (ie a Type II substrate), and fluorescence of the sample of step (i) is less than that of the control sample, then this may be indicative of cancer, or of sensitivity to platinum-based therapies. Sensitivity to

platinum-based therapies indicates a higher likelihood of a successful outcome of treatment with the platinum-based therapy and therefore a better prognosis. Platinum based drugs are major anticancer agents used in testicular and ovarian cancer and it is known that a lack of sensitivity to such drugs may indicate an unfavourable or unsuccessful outcome and a poor prognosis for the patient.

Where the polynucleotide substrate being used is a type I substrate (ie, a substrate for an AP endonuclease enzyme), and the fluorescence of the sample of step (i) is greater than that of the control sample, then this may be indicative of a lack of sensitivity to radiotherapy and bleomycin.

It will be appreciated that a diagnosis and prognosis may be made at the same time.

In a further embodiment, the assay may be used in a method to determine the sensitivity of a patient to a platinum based therapy, wherein the method comprises the steps

(i) incubating a sample containing cells from the patient with a polynucleotide according to the first aspect of the invention;
(ii) detecting fluorescence; and
(iii) comparing the fluorescence of step (ii) with that produced by a control sample.

Preferably, the polynucleotide is a type II polynucleotide as described above (ie a substrate for an XPG-like enzyme).

As outlined above, a lower level of XPG-like enzyme activity (indicated by a lower fluorescence) in the sample from the patient compared to the control sample is indicative of sensitivity to platinum based therapeutics.

The invention also provides a use of the polynucleotides of the first aspect of the invention in the above methods of diagnosing or prognosing cancer in a patient or determining the sensitivity of a patient to a platinum based therapy.

A third aspect of the invention provides a method of identifying an agent which modulates the activity of a DNA modifying enzyme the method comprising the steps of carrying out the assay of the second aspect of the invention in the presence of a test agent and comparing the fluorescence of step (ii) with that produced by the same enzyme and polynucleotide in the absence of the test agent.

The method comprises the steps of

(i) incubating the enzyme with a polynucleotide according to the first aspect of the invention in the presence of a test agent;
(ii) detecting fluorescence; and
(iii) comparing the fluorescence of step (ii) with that produced by the same enzyme and polynucleotide in the absence of the test agent.

The test agent may be any test agent which it is desired to investigate. Suitable agents include any known agents which are available commercially. Conveniently, the agent is one of a combinatorial library of compounds.

The modulation may be an increase or a decrease in the activity.

The enzyme may be any suitable DNA modifying enzyme. Preferably the enzyme is any one of an AP endonuclease, an XPG-like enzyme such as XPG or XPF-ERCC1, or a DNA glycosylase. Preferably the DNA glycosylase is 3AAG or Nth1.

Thus, this aspect of the invention includes methods of identifying agents which modulate the activity of an AP endonuclease or an XPG-like enzyme or a DNA glycosylase.

Suitable substrates for each enzyme type are described above in relation to the second aspect of the invention.

Where the enzyme used in the method is a DNA glycosylase, then as discussed above in relation to the second aspect of the invention, and depending on the type of substrate used, the method may further comprise the step of cleaving the polynucleotide at any abasic residues before detecting the fluorescence, as described above.

Conveniently, the components of step (i) are combined in an order such that the polynucleotide or the enzyme is added to the incubation mixture last, such that the enzyme does not have opportunity to act on the substrate (polynucleotide) in the absence of the test agent. Preferably, in step (i) the test agent is contacted with the enzyme and subsequently the polynucleotide (substrate) is added.

The optimal incubation time before detection of any resulting fluorescence may be determined by routine experimentation using the polynucleotide and enzyme added in the absence of a test agent.

Suitable methods for detecting the resulting fluorescence are described above in relation to the second aspect of the invention.

The comparison of the fluorescence produced by the same polynucleotide and enzyme in the presence and absence of the test agent allows the determination of whether the test agent is modulating the enzyme or not. Test agents which are found to modulate enzyme activity may be selected for further study. As described below, agents which inhibit the enzyme activity may be useful as anticancer agents. Thus, it is particularly preferred to identify test agents which are inhibitors. For example, in relation to determining whether a test agent is an inhibitor of the enzyme, if the fluorescence produced in step (ii) is less than that produced by the enzyme and polynucleotide in the absence of the test agent, then the test agent is an inhibitor of the activity of the enzyme. Preferably, agents are selected following the assay which cause a reduction in the fluorescence emitted of at least 10%, 20% or 30%. More preferably, the test agent selected for further study is one capable of inhibiting the enzyme such that the fluorescence is decreased by at least 40%, 50%, 60% or 70%, or 80%. The test agents selected may be ones which reduce the fluorescence generated by the activity of the enzyme being assayed by more than 90%, or prevents substantially any fluorescence being generated.

It may be useful to investigate the effect of the test agent on the activity of the DNA modifying enzyme at more than one concentration of test agent. It is preferred if a relatively low concentration of test agent is tested, since

modulators of enzyme activity which are effective at low concentration may represent more potent compounds. Preferably, the test agent is tested for inhibition or stimulation of the DNA modifying enzyme at a concentration of 50 μmoles/L or below.

Any test agent which is identified as a modulator (ie an inhibitor or a stimulator) of a DNA modifying enzyme may be selective for a particular enzyme type, such as for AP endonucleases, or for a specific enzyme, such as HAP1, or for a specific activity of a particular enzyme or enzyme type, such as the endonuclease activity of AP endonucleases rather than the exonuclease activity of these enzymes. Alternatively, the modulator may not show any selectivity, and may be a general inhibitor or stimulator of a type or activity of DNA modifying enzymes. Such selective modulators include compounds or agents that, at the same concentration, are at least twice as effective (eg at least four times as effective) at inhibiting or stimulating the activity of the DNA modifying enzyme for which they are selective as they are at inhibiting or stimulating the activity of another DNA modifying enzyme (eg the restriction enzyme HpaII).

Hence, it is preferred if the selectivity of any agent identified as being a modulator of a DNA modifying enzyme is further tested for its selectively and specificity. Methods of testing the selectivity of an agent are known in the art.

In an alternative embodiment of this aspect of the invention, the comparison of the fluorescence of step (ii) is with that produced by the same enzyme and polynucleotide and test agent in the presence of an additional enzyme which is capable of completely degrading DNA. An example of such an enzyme is DNAse I.

Hence, the method of this embodiment comprises the steps of

(i) incubating the enzyme with a polynucleotide according to the first aspect of the invention;

(ii) detecting fluorescence; and

(iii) comparing the fluorescence of step (ii) with that produced by the same enzyme and polynucleotide and test agent in the presence of a DNA degrading enzyme.

The assay and identification methods of the invention as described above are useful in the laboratory analysis of the function of DNA modifying enzymes, but are particularly useful as high-throughput methods of screening and analysis. The incubation mixture may be prepared and incubated in vessels such as microtitre plates and the preparation process automated using known automated dispensing. Since the methods are fluorescence based, they are also amenable to automated analysis machinery and tools and require only a minimal level of manual input. Methods of high throughput analysis are known in the art.

Agents identified or identifiable by the said methods may be useful as drug-like compounds or may be useful as lead compounds in the development of agents which are suitable for use in the treatment of certain diseases as discussed in more detail below. Thus, conveniently, test agents which modulate enzyme activity and which have the characteristics of a drug-like compound or lead compound are selected for further study.

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that

may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 1000 Daltons. Estimation of the solubility of drug-like compounds is described in Lipinski *et al* (2001) *Adv. Drug Deliv. Rev.* 46:3-26. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that these features are not essential. Hence, the agent identified as a modulator of a DNA modifying enzyme may be used as a lead compound to identify other compounds with similar modulatory activity but which also have desirable physical properties which makes them more suitable as drugs. The desirable properties depend on the intended use, but generally relate to the solubility, resistance to metabolism, effective concentration, bioavailability and lack of interaction with other medicaments or pharmaceutical carriers.

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the agent, whilst itself may or may not be suitable for use as a drug (for example it may not be suitable because it is only insufficiently potent against its intended target, insufficiently-selective in its action, unstable, poorly soluble, difficult to synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

It will be appreciated that the screening method of the invention may be used in retesting drug-like compounds and lead compounds. Also, the agents identified or identifiable by the screening methods as ones which

modulate enzyme activity may be put in further screens of their efficacy as, for example, anticancer agents. Thus, the screening assays of the invention may be combined with other screens for suitability of the agents as medicines, and for their efficacy in cancer treatment.

It will be understood that it will be desirable to identify compounds that may modulate the activity of the DNA modifying enzyme *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the interactions between the said enzyme and its substrate are substantially the same as *in vivo*.

The lead compound or drug-like compound may be selected for its particular attributes.

In one embodiment, the compound decreases the activity of said DNA modifying enzyme. For example, the compound may bind substantially reversibly or substantially irreversibly to the active site of said enzyme. In a further example, the compound may bind to a portion of said enzyme that is not the active site so as to interfere with the binding of the said enzyme to its substrate. In a still further example, the compound may bind to a portion of said enzyme so as to decrease said enzyme's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said enzymes's activity, for example in the activation of the said enzyme by an "upstream activator".

In a further embodiment, the agent or compound increases the activity of the DNA modifying enzyme. For example, the agent or compound may bind to a portion of said enzyme that is not the active site so as to aid the binding of the said enzyme to its substrate. In a still further example, the

compound may bind to a portion of said enzyme so as to increase said enzyme's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said enzyme's activity for example in the activation of the said enzyme by an "upstream activator".

Agents which stimulate DNA modifying enzymes may induce increased levels of DNA repair, which may be generally desirable to protect genomic DNA, or may be desirable in cases where an individual has come into contact with a known carcinogen or is known to be impaired in specific DNA repair processes. In addition, such compounds may protect the stem cells of an individual undergoing anti-cancer therapeutic regimes.

Preferably, a compound is selected which decreases the activity of the enzyme. More preferably, the compound substantially eliminates the activity of the enzyme. Compounds which inhibit DNA modifying enzymes may be useful as anticancer agents. Whether or not a compound decreases the activity a DNA modifying enzyme may be measured using the methods described above.

A fourth aspect of the invention provides a use of a polynucleotide according to the first aspect of the invention for assaying a DNA modifying enzyme.

Preferred forms of the polynucleotide are as described above in respect of the first aspect.

A fifth aspect of the invention provides an agent selected by the method of the third aspect of the invention as a modulator of the activity of a DNA

modifying enzyme. Preferably, the agent is selected as an inhibitor of an AP endonuclease or DNA glycosylase or XPG-like enzyme.

Preferably, the selected agents of the invention are low molecular weight agents. When used herein, the term "low molecular weight" includes compounds having a molecular weight of below 5000 g/mole, such as below 4000 g/mole (e.g. below 3000 g/mole), and particularly compounds having a molecular weight below 2500 g/mole (e.g. below 1500, 1200, 1000, 900 or, especially, 800 g/mole).

Agents identified or identifiable as inhibitors of an enzyme by the screening methods of the invention are likely to be useful laboratory reagents in the study of DNA-modifying enzymes. Such agents, especially when inhibitors of the enzyme, may also be useful in the treatment of disease where inhibition of the enzyme is desirable. In the case of HAP1 at least, inhibitors of this enzyme are likely to be useful in the treatment of cancer.

Inhibitors of AP endonucleases other than HAP1, DNA glycosylases and XPG-like enzymes may also be useful in treating cancer in an individual by sensitising cells against DNA damaging agents.

As discussed above, the agent may be selective or non-selective for a particular enzyme type (eg AP-endonuclease) or specific enzyme (eg HAP1). Preferably, the agent is selective for a particular enzyme type and more preferably, the agent is selective for a specific enzyme.

As described above, agents identified by the method of the third aspect of the invention may be useful as lead compounds in the development of compounds which are effective for use in the treatment of disease.

Either an agent identified by the method of the third aspect of the invention, or a compound developed from the said agent may be useful in combination with a cancer therapeutic agent such as a DNA damaging agent, wherein administration of the combination allows a lower does of the cancer therapeutic agent to be used without compromising on the therapeutic effect.

A sixth aspect of the invention provides a composition comprising an agent identifiable by the method of the third aspect of the invention and a cancer therapeutic agent. The agent may also be one according to the fifth aspect of the invention, which was identified by the identification methods described herein.

Preferably, the agent is a low molecular weight molecule as defined above.

The agent may be an agent that inhibits or stimulates the activity of any DNA modifying enzyme. Preferably the agent is one which inhibits the activity of such an enzyme. Preferably the enzyme is any one of an AP endonuclease or an XPG-like enzyme or a DNA glycosylase. More preferably, the agent is one that inhibits the activity of HAP1 or Nth1. Still more preferably, the agent is one which is selective for the enzyme or enzyme activity.

It is also preferred if the agent is one which is identified as being effective at low concentration, such as at less than 50μmoles/L.

It will be appreciated that the agent may be one which, after having been identified as a modulator of a DNA modifying enzyme as described above,

has been altered in terms of its chemical structure to improve its drug-like properties. For example, the agent identifiable by the method of the third aspect of the invention may have been used as a lead compound to determine a structure of the agent which has the desired solubility and bioavailability properties. Such modified or altered agents are included in the term "agent identifiable by the method of the third aspect of the invention" as used herein.

It will be appreciated that the composition may include either a single agent which inhibits one type of DNA modifying enzyme such as an AP endonuclease or a DNA glycosylase, or may include more than one such inhibitory agent, each such inhibitory agent inhibiting a different DNA modifying enzyme.

For example, the composition may comprise an agent identified as an inhibitor of HAP1 and a cancer therapeutic agent. The said composition may further comprise an inhibitor of a DNA glycosylase or an XPG-like enzyme, and a cancer therapeutic agent.

The cancer therapeutic agent is conveniently a chemical DNA damaging agent, such as one described in more detail below.

A seventh aspect of the invention provides a pharmaceutical composition comprising an agent identifiable by the method of the third aspect of the invention and a pharmaceutically acceptable carrier.

Preferably, the agent is a low molecular weight molecule as defined above.

By "pharmaceutically acceptable" is included that the formulation is sterile and pyrogen free. Suitable pharmaceutical carriers are well known in the art of pharmacy.

The composition of this aspect may comprise more than one agent which is identifiable by the method of the third aspect of the invention, the additional agent or agents differing in the nature of the enzyme it or they inhibit. For example, the pharmaceutical composition may be an agent identified as an inhibitor of an AP endonuclease such as HAP1 and a pharmaceutical carrier and may further comprise an agent which is identifiable as an inhibitor of a DNA glycosylase such as Nth1. Said composition may alternatively (ie instead of the DNA glycosylase inhibitor), or still further (ie in addition to the DNA glycosylase inhibitor), comprise an agent which is identifiable as an inhibitor of XPG or XPA.

In one embodiment of this aspect of the invention, the composition further comprises a cancer therapeutic agent. The cancer therapeutic agent is conveniently a chemical DNA damaging agent, such as one described in more detail below.

An eighth aspect of the invention provides an agent identifiable by the method of the third aspect of the invention or a composition of the invention for use in medicine.

Preferably, the agent is a low molecular weight molecule as defined above.

It is believed that the susceptibility of cells to cell death may be increased by inhibiting the activity of DNA modifying enzymes such as HAP1. This increased susceptibility may be desirable where the cells are tumour cells in

a patient. One way of exploiting this susceptibility is to expose the same cells to both agents that induce DNA damage, such as a DNA damaging agent as described below, and to an inhibitor of DNA repair, such as an inhibitor of HAP1. Thus, agents that inhibit DNA modifying enzyme activity as defined above, particularly low molecular weight agents, may be useful in medicine.

In addition to being useful in the treatment of cancer, agents which inhibit the activity of a DNA modifying enzyme as defined in the third aspect of the invention may be useful in the treatment of other diseases or conditions. For example, it is known that bacterial cells (e.g. *E. coli* cells) lacking the enzymes EndoIV and ExoIII are very sensitive to killing by nitric oxide. As this latter agent is produced by macrophages of the human immune system, inhibitors of AP endonuclease enzymes (such as EndoIV and ExoIII) may be useful as anti-microbial agents (e.g. anti-bacterial, anti-parasitic, anti-viral and/or anti-fungal agents).

A ninth aspect of the invention provides the use of an agent identifiable by the method of the third aspect of the invention in the manufacture of a medicament for treating cancer.

Preferably, the agent is a low molecular weight molecule as defined above.

As described above, it is believed that inhibitors of DNA modifying enzymes such as HAP1 may increase the susceptibility of cells to cell death. In cancer, many therapeutic approaches are aimed at killing the cancerous cells, or at least decreasing the rate of proliferation. Hence, an agent which promotes susceptibility to cell death may be useful in the treatment of cancer in a patient.

According to a preferred embodiment, the medicament is for treatment of cancer in a patient who is administered a cancer therapeutic agent such as a DNA damaging agent. DNA damaging agents are well known in the art of cancer therapy, and include ionising radiation and chemical agents such as those given below.

As discussed above, administration of the agent identified by the third aspect of the invention to a patient who is administered a cancer therapeutic agent such as a DNA damaging agent may allow a lower dose of the DNA damaging agent to be used without reducing the therapeutic effect. This is advantageous in decreasing any side effects which may be associated with use of the cancer therapeutic agent.

The term "administered" as used herein includes administration of the DNA damaging agent prior to, during or after treatment of the patient with the medicament that is prepared with the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention. Administration of the DNA damaging agent preferably takes place within the period of 48 hours before and/or within 48 hours after treatment with this medicament. It is particularly preferred if the administration occurs within 24 or, more preferably, 12 hours, before and/or after treatment with this medicament. Still more preferably, administration of the DNA damaging agent takes place within 6 hours, or 3 hours before and/or after treatment. It will be appreciated that the DNA damaging agent may be administered both before and after treatment with the medicament of the present invention.

Administration of multiple doses of the DNA damaging agent and/or the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention are also contemplated. In such cases, the relative time scales mentioned above relate to the time separation between administration of neighbouring doses of DNA damaging agent and the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention. For example, a single dose of the DNA damaging agent may be administered between two doses of the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention, which two doses are separated by up to 96 hours (e.g. by up to 24 hours, such as up to 6 hours). Further, administration of multiple doses of the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention during a period of continuous administration of a DNA damaging agent (e.g. during continuous radiation therapy such as during radio-immunotherapy and brachytherapy) are also contemplated.

Where multiple doses of DNA damaging agent are administered, the agent may or may not be the same at each administration. Further, where multiple doses of the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention may or may not be the same at each administration.

Where the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention is administered first to a patient, a number of different assay techniques may be used to determine the optimum time to administer the DNA damaging agent. For example, when the agent is an inhibitor of an AP endonuclease, the activity level of a AP endonuclease enzyme in the patient (either in tumour or other tissue) may

be monitored. In such cases, the DNA damaging agent may be administered once the AP endonuclease enzyme activity level drops below a predetermined value. Alternatively, the plasma concentration of the agent which inhibits the activity of a DNA modifying enzyme as defined in the third aspect of the invention may be monitored, in which case the DNA damaging agent may be administered at a predetermined point in the plasma concentration profile.

In order to monitor the effectiveness of the method of cancer treatment according to the invention (described below), the number and/or physical distribution of abasic sites may be monitored during and/or after treatment. Such monitoring may be achieved, for example, by using a suitable probe for abasic sites, such as that disclosed in Atamna, H. *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97(2), 686-691.

When the DNA damaging agent and the medicament are administered at the same time, they may either be co-administered as separate formulations or administered together in a single combined formulation.

In any of the foregoing aspects of the invention, where a DNA damaging agent that is radiation is administered to a patient, it may be administered by any method known to those skilled in the art. Such methods include administration by:

1) external beam (e.g. targeted X-ray source);
2) brachytherapy (i.e. sealed or unsealed sources inserted into or near the tumour site); and
3) targeted therapy (e.g. radioimmunotherapy using, for example, a radiolabelled antibody).

The agent may be any agent identifiable by the method of the third aspect of the invention. Preferably the agent is an inhibitor of the DNA modifying enzyme. More preferably the agent is a low molecular weight agent, as defined above.

Preferably, the agent is selective for an enzyme or enzyme activity as defined above, and also preferably, the agent is one which is identifiable as being effective at low concentration, as defined above.

The agent may be the sole active ingredient in the medicament, or may be one of several active ingredients. Where the medicament further comprises other active ingredients, another agent which is identifiable by the method of the third aspect of the invention may be included. In this case, it is preferred if the additional agent inhibits a different DNA modifying enzyme. Alternatively or additionally, a DNA damaging agent which is a chemical (rather than ionising radiation, for example) may be one of the other active ingredients.

In one embodiment, this aspect of the invention provides the use of a combination of a chemical DNA damaging agent and an agent identifiable by the method of the third aspect of the invention in the manufacture of a medicament for treating cancer. Thus, the chemical DNA damaging agent and the agent identifiable by the method of the third aspect of the invention may be combined in the same medicament before administration to the patient.

In one embodiment, the DNA damaging agent is one that induces the production of an AP site in DNA. According to this embodiment, it is preferable if the medicament of the present invention is manufactured with

an agent which is an inhibitor of AP endonucleases. It is especially preferred if the agent in the medicament is one which inhibits the HAP1 enzyme. It is still further preferred if the agent is selective for HAP1.

It is known that mammalian AP endonuclease enzymes are involved in the cellular protection of tumour cells against hypoxic stress (see, for example, Walker *et al* **(1994)** *Nucleic Acid Res.* 22:4484-4489. Thus, in another preferred embodiment of the tenth aspect of the invention, there is provided a composition comprising:

(a) an anti-angiogenesis agent, as hereinbefore defined; and

(b) an inhibitor of a mammalian AP endonuclease identifiable by the method of the third aspect of the invention.

As used herein, the term "DNA damaging agent" includes all agents that induce the production of an AP site in DNA. Particularly useful DNA damaging agents include ionising radiation (e.g. subatomic particle radiation such as α-particles, β-particles, neutrons, protons, mesons and heavy ions or electromagnetic radiation such as high-frequency X-rays or gamma rays) and the following chemical DNA damaging agents:

(a) Alkylating agents including:

(i) nitrogen mustards such as mechlorethamine (HN_2), cyclophosphamide, ifosfamide, melphalan (L-sarcolysin) and chlorambucil;

(ii) ethylenimines and methylmelamines such as hexamethylmelamine, thiotepa;

(iii) alkyl sulfonates and thiosulfonates such as busulfan, methyl methanesulfonate (MMS) and methyl methanethiosulfonate;

(iv) nitrosoureas and nitrosoguanidines such as carmustine (BCNU), lomustine (CCNU), semustine (methyl-CCNU), streptozocin (streptozotocin) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG); and

(v) triazenes such as dacarbazine (DTIC; dimethyltriazenoimidazole-carboxamide).

(b) Antimetabolites including:

(i) pyrimidine analogues such as fluorouracil (5-fluorouracil; 5-FU), floxuridine (fluorodeoxyuridine; FUdR) and cytarabine (cytosine arabinoside); and

(ii) purine analogues and related inhibitors such as mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG) and pentostatin (2'-deoxycoformycin).

(c) Natural Products including:

(i) epipodophyllotoxins such as etoposide and teniposide; and

(ii) antibiotics such as dactinomycin (actinomycin A, C, D or F), daunorubicin (daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin) and mitomycin (mitomycin A, B or C).

(d) Miscellaneous agents including:

(i) platinum coordination complexes such as cisplatin (*cis*-DDP) and carboplatin;

(ii) anthracenedione such as mitoxantrone and anthracycline;

(iii) substituted urea such as hydroxyurea;

(iv) methyl hydrazine derivatives such as procarbazine (N-methylhydrazine, MIH);

(v) photoactivatable compounds (e.g. psoralens); and

(vi) DNA topoisomerase inhibitors (e.g. m-amsacrine and camptothecin).

The term "cancer therapeutic agent", when used herein includes any compound that can be used to treat cancer. The term thus includes the above-listed DNA damaging agents and further comprises: antimetabolites including folic acid analogues such as methotrexate (amethopterin); natural products including vinca alkaloids such as vinblastine (VLB) and vincristine, enzymes such as L-asparaginase, biological response modifiers such as interferon alphenomes; miscellaneous agents including adrenocortical suppressants such as mitotane (*o,p'*-DDD) and aminoglutethimide, taxol and analogues/derivatives, hormone agonists/antagonists such as flutamide and tamoxifen, anti-angiogenesis agents (eg SU6668, SU5416, combretastatin A4, angiostatin and endostatin) and immunotherapeutic agents (e.g. radiolabelled Bexxar™ and Theragyn™ (Pemtumomab™) antibodies).

The term "cancer" will be well understood by those skilled in the art, and includes any form of malignancy or premalignancy. Cancers that may be mentioned include those that demonstrate enhanced expression of DNA repair enzymes, such as the human carcinoma cell lines described in: Lai *et al. Biochem. Pharmacol.* 37, 4597-4600 (**1988**); Hospers *et al. Cancer Res.* 48, 6803-6807 (**1988**); Masuda *et al. Cancer Res.* 48, 5713-5716 (**1988**); Kraker *et al. Cancer Lett.* 38, 307-314 (**1988**); and Scanlon *et al. Anticancer Res.* 9, 1301-1312 (**1989**), the disclosures of which documents are hereby incorporated by reference. Further cancers that may be mentioned include leukemias, lymphomas, myelomas, neuroblastomas, neoplasias of bladder, testicular, endometrial, gastric or lung origin neoplasias. Particular cancers that may be mentioned include the following neoplasias: Hodgkin's, non-Hodgkin's and Burkitt's lymphomas; myelomas; glioblastomas, medulloblastomas and neuroblastomas; pancreatic islet cell carcinomas;

osteogenic sarcoma; breast, endometrial, testicular, cervical, gastric, squamous cell, adrenocortical and small cell lung carcinomas and the like.

The compositions according to the invention will normally be administered orally, subcutaneously, intravenously, intraarterially, transdermally, intranasally, by inhalation, or by any other parenteral route, in the form of pharmaceutical preparations comprising the relevant active ingredient(s) either as such or in the form of (a) non-toxic organic or inorganic acid or base addition salt(s), in (a) pharmaceutically acceptable dosage form(s). Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions or separate components or medicaments may be administered at varying doses.

The medicament will normally be administered orally, subcutaneously, intravenously, intraarterially, transdermally, intranasally, by inhalation, or by any other parenteral route, in the form of pharmaceutical preparations comprising the relevant active ingredient(s) either as such or in the form of (a) non-toxic organic or inorganic acid or base addition salt(s), in (a) pharmaceutically acceptable dosage form(s). Depending upon the disorder and patient to be treated, as well as the route of administration, the compositions or separate components of the medicament may be administered at varying doses.

The agents, compositions, and the separate components of the medicament, according to the invention are preferably formulated for use in medicine (e.g. in admixture with a pharmaceutically acceptable adjuvant, diluent and/or carrier). Such formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association

the relevant active ingredient(s) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient(s) with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g. sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

Suitable doses of DNA modifying enzyme inhibitors such as HAP1 inhibitors, and/or (chemical) DNA damaging agents (as appropriate), in any

of the above-mentioned cancer treatments or medicaments may be determined routinely by the medical practitioner or other skilled person.

A tenth aspect of the invention provides the use of an agent identifiable by the method of the third aspect of the invention in the manufacture of a medicament for treatment of a condition where inhibition of a DNA modifying enzyme is required.

Preferably, the enzyme is an AP endonuclease, XPG-like enzyme, or a DNA glycosylase. Where the enzyme is an AP endonuclease, it is preferred if it is HAP1.

It will be appreciated that the agent identifiable by the method of the third aspect of the invention will be one which inhibits the enzyme whose inhibition is desired. For example, where inhibition of HAP1 is desired, the agent will be one which is identifiable by the method of the third aspect as being an inhibitor of the HAP1 enzyme.

An eleventh aspect of the invention provides a method of treating cancer in a patient comprising administering to the patient an agent identifiable by the method of the third aspect of the invention.

Preferably, the agent inhibits the activity of a DNA modifying enzyme. Preferably, the agent is a low molecular weight molecule, as defined above.

The medicament of the invention described above or therapeutic system of the invention described below may be useful in the method of this aspect of the invention.

In one embodiment, the patient is administered a DNA damaging agent, as described above. The patient may be administered the DNA damaging agent before, at the same time as, or after administration of the agent identifiable by the method of the third aspect of the invention. By "administered", we include the meaning as defined above.

DNA damaging agents are described in more detail above, and include chemical agents and ionising radiation. Preferably, the DNA damaging agent induces the production of AP sites (abasic sites) in DNA and preferably the agent identifiable by the method of the third aspect of the invention is one which inhibits an AP endonuclease or a DNA glycosylase.

A twelfth aspect of the invention provides a method of inhibiting the activity of a DNA modifying enzyme comprising contacting the enzyme with an agent identifiable by the method of the third aspect of the invention.

Preferably, the enzyme is an AP endonuclease, XPG-like enzyme, or a DNA glycosylase. Where the enzyme is an AP endonuclease, it is preferred if is HAP1.

In a preferred embodiment, the enzyme is in a cell, and the cell is in a patient. Hence, contacting the enzyme with the agent may be by means of administration of the agent to the patient. The medicament or therapeutic system of the invention described above and below respectively may be useful in the method of this aspect of the invention.

Where the enzyme is in a cell in a patient, it is preferred if the cell is a malignant or premalignant cell.

The patient is preferably a patient who has a condition where inhibition of the activity of a DNA modifying enzyme such as an AP endonuclease, an XPG-like enzyme, or a DNA glycosylase is desired.

Means of administering an agent to a patient are described above.

As well as being useful in the treatment of cancer, inhibition of mammalian DNA modifying enzymes may also be desirable in other circumstances. For example, patients suffering from chronic inflammatory and oxyradical overload diseases (such as ulcerative colitis, viral hepatitis, Wilson disease, haemochromatosis, chronic gastritis, chronic pancreatitis and Barret oesophagus), which conditions are linked with an increased susceptibility to cancer, may benefit from the administration of a mammalian AP endonuclease inhibitor. Further, inhibition of mammalian AP endonucleases may be desirable in the treatment of Alzheimer's disease, which is associated with senile plaques, plaque-like structures and areas of brain injury that demonstrate elevated HAP1 expression.

A thirteenth aspect of the invention provides a therapeutic system comprising an agent identifiable by the method of the third aspect of the invention and a cancer therapeutic agent.

For the avoidance of doubt, the therapeutic system is effectively a kit of parts for use in therapy.

Agents for inclusion in the system are as described above. Preferably, the agent is a low molecular weight molecule as defined above and preferably, is one which inhibits the activity of any one of an AP endonuclease, an

XPG-like enzyme, or a DNA glycosylase. Where the enzyme is an AP endonuclease, it is preferred if it is the enzyme HAP1.

Preferably, the cancer therapeutic agent is a DNA damaging agent as described above. More preferably, the agent is a chemical agent as described above.

The therapeutic system may contain the said components packaged and presented in suitable formulations for use in combination, either for administration simultaneously or sequentially (including sequential administrations which are separated in time).

An alternative utility for the agents identifiable by the third aspect of the invention as being inhibitors of a DNA modifying enzyme, which may be AP endonuclease inhibitors, is in the production of mammalian (preferably human) cells which can be used in mutagenic, cytostatic or cytotoxic testing. That is, test cells (preferably of human origin) may be generated by contacting them with one or more of the said inhibiting agents. Such test cells may then be used either:

(a) in a method of detecting the mutagenic, cytostatic or cytotoxic nature of a compound, by monitoring the frequency of phenotypic change in test cells in the presence and absence of said compound; or

(b) in a method of assessing the ability of a compound to protect against DNA damage, by monitoring the frequency of DNA damage, in the presence and absence of said compound, in groups of test cells that have been contacted with a known carcinogen.

As the crystal structures of HAP1 and other AP endonuclease enzymes are known (see, for example Barzilay, G. *et al.* (1995) *Nature Structural*

Biology 2:561-567; Gorman *et al* (1997) *EMBO J.* 16:6548-58), agents that are known to inhibit DNA modifying enzymes such as AP endonucleases (eg agents identifiable by the method of the third aspect of the invention) may be analysed in relation to such crystal structures. Using the results of such analyses, modifications of the structures of existing inhibitors, aimed at producing new and more potent inhibitors of AP endonucleases, may be proposed. Methods of identifying AP endonuclease inhibitors by such analyses, and the compounds thus identified, are also considered to be within the scope of the present invention.

Hence, a fourteenth aspect of the invention provides a method of detecting the mutagenic, cytostatic or cytotoxic nature of a test compound, which method comprises:

(i) preparing test cells by contacting cells with one or more agents which are inhibitors identifiable by the method of the third aspect of the invention;

(ii) in those test cells, monitoring the frequency of phenotypic change, the cell proliferation or the frequency of cell death (as appropriate) in the presence and absence of said test compound.

A fifteenth aspect of the invention provides a method of assessing the ability of a compound to protect against DNA damage, which method comprises:

(i) preparing test cells by contacting cells with one or more agents which are inhibitors identifiable by the method of the third aspect of the invention;

(ii) contacting those test cells with a known carcinogen; and

(iii) monitoring the frequency of DNA damage in those test cells in the presence and absence of said compound.

A sixteenth aspect of the invention provides a kit of parts comprising a polynucleotide according to the first aspect of the invention and a DNA modifying enzyme.

In a preferred embodiment, the enzyme is any one of an AP endonuclease, an XPG-like enzyme, or a DNA glycosylase.

The DNA modifying enzyme may be supplied in any convenient form, such as a polypeptide in an aqueous solution which is frozen or chilled. The enzyme may be provided as a means of producing the enzyme, such as the encoding nucleic acid, preferably comprised within a suitable expression vector. Where the enzyme is supplied as nucleic acid, it may be convenient to further include a means of expressing the nucleic acid in the kit. Suitable, and preferred means of protein expression include bacterial cells or mammalian or insect cells which have been transformed or transfected with the nucleic acid. Cell culture for protein expression, and isolation and purification of the expressed protein are standard techniques in the art of biochemistry. A further means of producing the protein is *in vitro* expression of mRNA encoding the enzyme using a translation system such as rabbit reticulocyte lysate (available from Promega).

The nucleic acid may be provided separately to the expression cell, or may be provided within the expression cell. Stably transfected or transformed cells are known in the art.

Where the kit provides a means for expressing the enzyme from a nucleic acid, or where the kit provides cells comprising the nucleic acid, the kit may usefully further provide a means of confirming the production of the enzyme. Such a means may be an antibody selective for the enzyme to be produced, for example; a HAP1 polyclonal antibody is available from Trevigen, 8405 Helgerman Court, Gaithersburg, MD 20877, an XPG monoclonal (8H7) is available from Abcam Ltd, 31 Cambridge Science Park, Milton Road, Cambridge CB4 0FX, UK, and 3AAG and hNth1 polyclonals from Novus-Biologicals are also supplied by Abcam. Typically, such an antibody would be detectably labelled, or suitable for conjugation to a detectable label.

Where the enzyme is a DNA glycosylase, then the kit may usefully further comprise an AP endonuclease such as the HAP1 enzyme or a chemical DNA cleaving agent such as a diamine or by other molecules as described in Constant *et al* (1990) *Anti-Cancer Drug Design* 5:59-62. As described above, an additional enzyme may be provided as a polypeptide or as a means of producing the polypeptide.

Where the DNA glycosylase is the enzyme Nth1, the kit may further comprise the enzyme XPG or the protein YB-1, or both XPG and YB-1. As described above, XPG and YB-1 are believed to serve as a cofactor for the efficient function of Nth1 and XPG promotes binding of Nth1 to damaged DNA (Klungland *et al* (1999) *Mol. Cell* 3:33-42). The XPG and/or YB-1 may be provided in the kit as an active, wild type enzyme, or in the case of XPG, may be provided as a catalytic site mutant which is itself inactive in nucleotide excision repair. A suitable catalytic site mutant of XPG is described in Klungland *et al* (1999) *Mol. Cell* 3:33-42.

The kits of the invention may further comprise one or more multiwell plates such as 96 well microtitre plates, and/or the enzyme DNAase I. As described above, DNAase I may be useful in providing a positive control when testing whether a test agent is an inhibitor of the DNA modifying enzyme.

Such kits are useful for carrying out the assay of the second or third aspects of the invention

The invention will now be described with the use of the following Figures and Examples.

Figure 1.

Substrate Preparation. Substrate fluorescence was measured in 200µl samples in a spectramax Gemini plate reader at excitation wavelength (Ex) of 595 nm and emision wavelength (Em) of 535 nm. A cut off filter of 530nm was employed. Circles – HAP1 substrate. Squares – XPG substrate. For HAP1 assay, Ex 495 nm and Em 540 nm were used. Under XPG assay conditions, Ex 485 nm and Em 538 nm were used.

Figure 2.

HAP1 assay principle. Under the influence of HAP1, the fluorescent labelled DNA strand is cleaved at the AP site (abasic site) at the 5' end. The 5' end of the fluorescent labelled strand is now a 6 base oligonucleotide containing the fluorescent label (fluorescein). This oligonucleotide is now free to melt into solution depending upon the number and nature of the bases and the temperature. If the 5'-fluorescent oligonucleotide enters solution, then the quenching effect of the 3' quenching molecule (DABCYL) is lost and a fluorescent signal is registered.

Figure 3.

Fluorescent assay of HAP1 activity as described in Example 1. Measurement of HAP1 abasic site endonuclease activity: Points are the average of eight wells, error bars of one standard deviation. Closed circles: • abasic site substrate, Open circles: o no abasic site substrate.

Figure 4.

Effects of DNA length on fluorescent signal. Numbers along the bottom axis indicate the distance of an AP site (abasic site) from the 5' fluor labelled end.

Figure 5.

Reaction rates using substrates containing abasic sites located 5, 6 and 7 bases from the 5' labelled end at two temperatures. Open symbols represent experiments run at 25°C, solid symbols represent experiments run at 37°C. Triangles indicate an abasic site 5 bases from the 5' fluor labelled end, squares indicate an abasic site 6 bases from the 5' fluor labelled end and circles indicate an abasic site 7 bases from the 5' fluor labelled end.

Figure 6.

Substrate for XPG DNA modifying enzyme. This repair enzyme cleaves at the end of a non base-paired section of DNA. In this case the quench fluor pair is kept together directly by its own base-pairing and indirectly via the other end of the DNA molecule. Upon action by XPG the fluor labelled strand is free to diffuse into solution to give a fluorescent signal. Possible cleavage sites are indicated within the substrate. However, the precise cleavage sites have not been determined but are in positions such that upon

cleavage the fluor and quench are separated leading to an increase in fluorescence.

Figure 7.

Substrates for DNA glycosylases.

a) HAP1 type substrate. This assay is essentially identical to the HAP1 assay described in Figure 2, except that the abasic site is generated by a DNA glycosylase acting upon a modified base.

b) Multi-substrate for glycosylases. As the glycosylase enzyme converts damaged bases to abasic sites, this removes base pairs and therefore lowers the melting temperature of the DNA molecule. If at the experimental temperature the DNA melts then a fluorescent signal will register.

Figure 8.

Increase in fluorescence measured with time in XPG treated wells when compared to DNA only wells. Experiments were in triplicate and error bars are of one standard deviation.

Figure 9.

Figure 9 shows cleavage of a Type II substrate by AAG. Solid symbols represent linear substrate (negative control), Open symbols represent AAG substrate. The AAG substrate is cleaved by the diamine at an abasic site subsequent to AAG removal of the inosine base and fluor is released to give signal.

Figure 10.

Figure 10 shows experiments performed at various AAG concentrations with both AAG substrate and linear substrate (negative control). The rates

of reaction are plotted against enzyme concentration. Triplicates, error bars of 1 s.d.

Figure 11.

Figure 11 shows the results of a control experiment using AAG against a UDG melting substrate 1U.

Figure 12.

Figure 12 shows the result of an experiment using AAG against AAG melting substrate. In both Figures 11 and 12, solid symbols represent controls (no AAG present), and open symbols represent samples with 50nM AAG present.

Figure 13.

Figure 13 shows that UDG destabilises UDG melting substrates 1U and 2U. Open squares - 1U control, Solid squares – 1U plus UDG. Open circles – 2U control, Solid circles – 2U plus UDG.

Figure 14.

Figure 14 shows that XPG cleaves a bubble DNA substrate but not a linear DNA substrate. Open circles – linear substrate. Solid circles – bubble substrate.

Figure 15.

Figure 15 shows that XPF/ERCC1 cleaves a bubble DNA substrate, but not a linear DNA substrate. Triangles – Linear substrate, Squares – bubble substrate.

Example 1

Measurement of HAP1 abasic site endonuclease activity

The substrate illustrated in Figure 2 was annealed in HAP1 assay buffer (HAB: 50mM Tris-HCl, pH7.5, 50mM NaCl, 10mM $MgCl_2$, 2 mM dithiothreitol,) and 100 µl was added to a series of 96-well microplate wells. 50µl of HAB was added containing 250 nM of HAP1 DNA substrate with either an abasic site 7 bases from the 5' fluor labelled end (as illustrated in Figure 2) or an equivalent DNA substrate lacking an abasic site. The plate was warmed to 37°C (10 minutes) in a microplate reader (Spectramax Gemini, Molecular Devices, CA, USA). 50µl of HAB was then added and contained either HAP1 (20ng; HAP1 can be obtained commercially from Trevigen Ltd. Gaithersburg, MD, USA or produced from *E.coli* expression systems and purified by affinity tag chromatography e.g. hexahistidine tag) or no HAP1 (control). The plate was mixed using the shake function on the plate reader and the fluorescence measured every 60 seconds.

The increase in fluorescence of the HAP1-containing well compared to the control well is plotted against time in Figure 3 for both substrates. The abasic substrate shows an increase in fluorescence (solid circles) where the equivalent double stranded DNA substrate lacking an abasic site shows little increase (open circles). The increase in fluorescence is attributed to the release of fluorescently-labelled 6 base oligonucleotide into solution after HAP1 cleavage of the substrate. Thus, the abasic site endonuclease activity of HAP1 is measured using the abasic substrate.

The substrate lacking an abasic site can be made to give a positive signal by adding the non specific DNA nuclease DNAase I (a DNA nuclease which will degrade the substrate fully and yield a maximal fluorescence value) or

the 3'-5' exonuclease Exo III. DNAase and Exo III are commercially available from Sigma and New England BioLabs, respectively.

Identical reactions may also be performed with test agents dissolved in 100µl HAP1 assay buffer (HAB) to identify compounds at concentrations of 5-20µM, that stimulate or inhibit AP endonuclease activity.

Example 2

Optimisation of abasic site position.

Experiments similar to that shown in Figure 3 and described in Example 1 were performed using substrates with an abasic site (X in Figure 2). Situated 5-10 bases away from a 5' fluor labelled base. Upon cleavage of the abasic site by HAP1, these substrates give rise to fluor labelled products of 4-9 bases in length.

Six sets of reaction mixtures were prepared in microplate wells as described in Example 1, each containing 100 nM of DNA substrate and 20 ng of HAP1. Each set was read for fluorescence of the DNA substrate alone before addition of HAP1 to one well and the DNAase (a non-specific DNA nuclease) to the other. Each set was incubated for 60 minutes at 25°C to allow reactions to run to completeness. The percentage of fluorescent signal seen in HAP1 treated wells, when compared to DNAase treated wells, is shown in Figure 4 (shaded bars). Substrates with the abasic site positioned 6-7 bases from the 5' fluor labelled end of the upper DNA strand give near maximal signal, whilst those containing the abasic site 8 or more bases from the 5' fluor labelled end give less signal and this decreases as the distance from the 5' fluor labelled end increases. The temperature of the reactions was then increased to 37°C for 30 minutes and the wells re-read

(non-shaded bars). The value for the % fluorescence in the substrates with abasic sites 8-10 bases from the 5-labelled end increases, presumably because the equilibrium of bound to unbound cleaved section decreases. The anomalous decrease in signal with an abasic site 5 bases from the 5' fluor labelled end is explained in Figure 5.

Determination of the optimal distance of the abasic site from the labelled DNA end.

To investigate the rates of reactions in substrates containing abasic sites 5, 6, and 7 bases from the 5' fluor labelled end three substrates were incubated with HAP1 at 25°C and 37°C. Reactions were carried out as described previously and each contained 100 nM of DNA substrate and 20 ng of HAP1. Figure 5 shows the difference in rates of the substrates due to temperature and abasic site position. With the abasic site 5 bases from the 5' fluor labelled end, the substrate is not cleaved efficiently by HAP1 and the rate is slow. (This would explain the low % release of fluorescence in Figure 4 with this substrate, i.e. it has not been fully cleaved). The substrates with the abasic site 6 or 7 bases from the 5' fluor labelled end both gave good signal.

The substrate with an abasic site 7 bases from the 5' fluor labelled end was chosen for further study at 37°C. HAP1 cleavage of this substrate was investigated under various buffer conditions and it was found that the most suitable buffer for reaction would fall in the following range (20 - 50mM Tris-HCl, pH 7.5 – 8.0, 1-100 mM NaCl, 0.1 – 1 mM $MgCl_2$, 2 mM dithiothreitol). All buffer components were purchased from Sigma chemicals (UK).

Example 3

Assay for XPG activity

XPG assay buffer (110 µl, 20mM Tris-Cl pH 8.0, 5mM $MgCl_2$, 1mM DTT,) containing 50 nM XPG substrate (as shown in Figure 6) was added to wells in a 96-well plate (Microfluor 2, Dynex) and the plate warmed for 10 minutes to 37°C in the plate reader (SpectraMax Gemini, Molecular Devices). The oligonucleotides were annealed as described above. Reactions were initiated by adding 10 µL XPG (produced from a baculoviral expression system termed BAC-TO-BAC Baculovirus Expression Systems supplied by Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, UK) in insect cell cultures and purified by affinity tag chromatography) in stock solution (50 µg/ml in 20 mM HEPES pH 7.4, 20% w/v glycerol). Fluorescence intensity 485/538 (Ex/Em) was measured at 37°C every ten minutes minute for one hour (Figure 8).

Example 4

Further substrates and assays DNA substrates used

DNA substrates used

Linear substrate (control)

```
5'     FAM-GCCCCCGGGGGACTGCCTGGCTTACTGCTCG      3'
3' DABCYL-CGGGGGCCCCCTGACGGACCGAATGACGAGC       5'
```

AAG substrate (Type III substrate)

```
5'     FAM-GCCCCCIGGGGACTGCCTGGCTTACTGCTCG      3'
3' DABCYL-CGGGGGTCCCCTGACGGACCGAATGACGAGC       5'
```

I = Deoxyinosine

UDG substrate (Type III substrate)

5' FAM-GCCCCCUGGGGACTGCCTGGCTTACTGCTCG 3'

3' DABCYL-CGGGGGCCCCCTGACGGACCGAATGACGAGC 5'

U = Deoxyuracil

AAG melting substrate (Type IV substrate)

5' FAM-GCTAGTCICTGGATG 3'

3' DABCYL-CGATCAGTGACCTACAC 5'

I = Deoxyinosine

UDG melting substrate 1U (Type IV substrate)

5' FAM-GCTAGTCUCTGGATG 3'

3' DABCYL-CGATCAGAGACCTAC 5'

U = Deoxyuracil

UDG melting substrate 2U (Type IV substrate)

5' FAM-GCTAGUCTCUGGATG 3'

3' DABCYL-CGATCAGAGACCTAC 5'

U = Deoxyuracil

Bubble substrate (Type II substrate)

5' FAM-CGCTCAA**CCCCCCCCCCCCCC**GCGCCACGTTGCTGCCC 3'

3'DABCYL-GCGAGTT**TTTTTTTTTTTTTT**CGCGGTGCAACGACGGG 5'

Bold letters indicate an unpaired 'bubble' region.

All experiments were performed in a Spectramax Gemini fluorescence plate reader (molecular devices). Excitation wavelength, 490 nm, Emission read at 540 nm, with a cutoff filter at 530 nm.

Enzymes

AAG – 3 alkyl adenine glycosylase – removes inosine bases from double stranded DNA to leave an abasic site.

UDG – uracil deglycosylase - removes uracil bases from double stranded DNA to leave an abasic site.

HAP1 – cleaves a single strand of double stranded DNA at an abasic site.

XPG – Cleaves a single DNA strand near the junctions of unpaired and paired bases of double stranded DNA.

XPF/ERCC1 - Cleaves a single DNA strand near the junctions of unpaired and paired bases of double stranded DNA. This enzyme cleaves on the opposite DNA strand to that of XPG.

Diamine

The diamine compound N,N'-dimethylethylene diamine (Sigma, UK) cleaves abasic DNA strands and does not cleave intact DNA.

AAG substrate is cleaved in the presence of AAG and diamine

AAG (100 nM) incubated with AAG substrate, 10 mM diamine and linear substrate 50 nM in a 384 well white Greiner microplate. 60 µl reaction volumes in AAG buffer (35 mM HEPES, pH 7.0, 25mM NaCl, 0.5 mM EDTA, 0.5 mM Dithiothreitol). Plate incubated for 1 hour at 37°C, the increase in fluorescence of test and control wells for each substrate is plotted in Figure 9. The rate of cleavage is dependent upon AAG concentration as shown in Figure 10.

AAG destabilises the AAG melting substrate

AAG (50 nM) incubated with AAG melting substrate (25 nM) and UDG melting substrate 1U (25 nM, negative control), in a 384 well white Greiner microplate.

60 µl reaction volumes in AAG buffer (35 mM HEPES, pH 7.0, 25mM NaCl, 0.5 mM EDTA, 0.5 mM Dithiothreitol).

Reactions were incubated for 1 hour at 37°C, the change in fluorescence with time of test and control wells for each substrate are plotted below.

Control Experiment AAG vs `UDG melting substrate 1U is shown in Figure 11.

AAG vs AAG melting substrate is shown in Figure 12.

In each figure, solid symbols represent controls (no AAG present), and open symbols represent samples with 50 nM AAG present.

AAG removes the inosine base from the AAG melting substrate and destabilises the substrate. The subsequent dissociation of the two strands of the substrate leads to an increase in free fluor and hence an increase in measured fluorescence. This effect is not seen in the control UDG melting substrate 1U, which is not a substrate for AAG as it contains uracil instead of inosine.

UDG destabilises UDG melting substrates 1U and 2U

UDG (0.1 units, Sigma, UK) was incubated with UDG melting substrates 1U and 2U (100nM), in a 96 well white Greiner microplate. Reaction volumes of 150 µl in UDG buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM Dithiothreitol). Plate incubated for 1 hour at 37°C, the change in fluorescence with time of test wells compared to control wells for each substrate are plotted in Figure 13.

UDG removes the uracil base(s) from the UDG melting substrates and destabilises these substrates. The subsequent dissociation of the two strands of the substrate leads to an increase in free fluor and hence an increase in measured fluorescence.

XPG cleaves a bubble DNA substrate but not a linear DNA substrates

XPG was incubated with bubble or linear DNA substrates (40 nM) at the concentrations indicated in a 384 well white Greiner microplate.

50 µl reaction volumes in XPG buffer (25 mM HEPES, pH 8.0, 10mM KCl, 2.5 mM $MgCl_2$, 1 mM Dithiothreitol).

Incubation was for 2 hours at 37°C, the rate of increase in fluorescence compared to DNA only controls for each substrate is plotted below against XPG concentration. Values are averages of duplicates, error bars of 1 s.d. (see Figure 14).

XPG is believed to cleave the bubble substrate (below) in the underlined regions and in any event releases fluor when cleaved. XPG does not cleave linear substrate, as this substrate does not have the correct tertiary structure.

5' FAM-CGCTCAACCCCCCCCCCCCCCGCGCCACGTTGCTGCCC 3'
3'DABCYL-GCGAGTTTTTTTTTTTTTTTTCGCGGTGCAACGACGGG 5'

Bold letters indicate an unpaired 'bubble' region.

XPF/ERCC1 cleaves a bubble DNA substrate, but not a linear DNA substrate

XPF/ERCC1 was incubated at the concentrations indicated with similar amounts (approx 50 nM) of linear substrate and bubble substrate for 45 minutes at 37°C. Assay volume was 200 µl of XPF buffer (25 mM HEPES, pH 8.0, 40 mM NaCl, 2 mM $MgCl_2$, 1 mM Dithiothreitol) in 96 well white Greiner microplates.

The rates of increase in fluorescence compared to DNA only controls for each substrate are plotted below vs XPF/ERCC1 concentration. Single point assays. See Figure 15.

XPF/ERCC1 is believed to cleave the DNA in the bubble substrate in the underlined regions and in any event releases fluor upon cleavage.

XPF/ERCC1 does not cleave linear substrate, as this substrate does not have the correct tertiary structure.

Bubble substrate

5' FAM-CGCTCAACCCCCCCCCCCCCCGCGCCACGTTGCTGCCC 3'

3'DABCYL-GCGAGTTTTTTTTTTTTTTTTCGCGGTGCAACGACGGG 5'

Example 5

Use of assays in high throughput drug screens

The assays described above are suitable for adaptation to high throughput screens due to, for example, the use of fluorescence, the possibility of performing the entire assay in a single well of a multi-well plate, the small volumes which can be used, the speed of readout of a result from the assay and the fact that time consuming steps such as gel electrophoresis are not required.

Means of adapting the assays described herein to a high throughput screen will be readily seen by the skilled person and will require at most only routine experimentation.

CLAIMS

1. A polynucleotide having a double stranded portion which is interrupted by at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair, the molecule further having attached thereto a fluorescent moiety and a quenching moiety which quenches the fluorescence of the fluorescent moiety.

2. A polynucleotide according to Claim 1 having a double stranded portion with one strand having attached thereto a fluorescent moiety and the other strand having attached thereto a quenching moiety which quenches the fluorescence of the fluorescent moiety wherein the double stranded portion is interrupted by at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair.

3. A polynucleotide according to Claim 1 or 2 which comprises two separate strands.

4. A polynucleotide according to any one of Claims 1 to 3 which has at least one cleavable bond in one strand of nucleic acid at or adjacent to the said at least one residue wherein said cleavable bond or bonds is in a position whereby cleavage of the said cleavable bond or bonds is sufficient to dissociate at least part of the double stranded portion of the polynucleotide such that quenching of the fluorescence of the fluorescent moiety by the quenching moiety is reduced.

5. A polynucleotide according to any one of Claims 1 to 4 wherein the polynucleotide is a substrate for any one of an AP endonuclease, XPG, XPF-ERCC1 or a DNA glycosylase.

6. A polynucleotide according to any one of Claims 1 to 5 wherein the at least one residue which does not participate in an A-T or G-C base pair is an abasic residue.

7. A polynucleotide according to Claim 6 wherein the abasic residue is a nucleic acid residue which contains tetrahydrofuran in place of a base.

8. A polynucleotide according to any one of Claims 1 to 5 wherein the at least one residue which does not participate in an A-T or G-C base pair does not have a complementary partner in the opposite strand.

9. A polynucleotide according to any one of Claims 1 to 5 wherein the at least one residue which does not participate in an A-T or G-C base pair has a damaged base.

10. A polynucleotide according to Claim 9 wherein the damaged base is 8-oxoguanine or formamidopyrimidine or inosine or uracil.

11. A polynucleotide according to any one of Claims 1 to 10 wherein there is more than one residue which does not participate in an A-T or G-C base pair.

12. A polynucleotide according to any one of Claims 1 to 11 wherein the residue which does not participate in an A-T or G-C base pair is located no more than 10 bases from a 5' terminus.

13. A polynucleotide according to any one of Claims 1 to 12 wherein the at least one fluorescent moiety is fluorescein.

14. A polynucleotide according to any one of Claims 1 to 13 wherein the quencher is DABCYL.

15. A polynucleotide according to any one of Claims 1 to 14 wherein the fluorescent and quenching moieties are located at adjacent 3' and 5' termini.

16. A method of assaying for the activity of a DNA modifying enzyme, the method comprising

(i) incubating the enzyme with a polynucleotide according to any one of Claims 1 to 15; and

(ii) detecting fluorescence.

17. A method according to Claim 16 wherein the enzyme cleaves the phosphodiester backbone of one strand of the polynucleotide.

18. A method according to Claim 17 wherein the enzyme is an AP endonuclease or an XPG-like enzyme.

19. The method according to Claim 16 wherein the enzyme converts at least one residue to an abasic residue.

20. A method according to Claim 19 wherein the enzyme is a DNA glycosylase.

21. A method according to Claim 19 or 20 further comprising the step of cleaving the phosphodiester backbone of one strand of the polynucleotide at any abasic residues before detecting fluorescence.

22. A method according to Claim 21 wherein the cleavage of the phosphodiester backbone of one strand of the polynucleotide as said is by adding an AP endonuclease or a chemical DNA cleaving agent, such as a diamine.

23. A method according to Claim 22 wherein the polynucleotide is one according to Claim 9 or 10.

24. A method of identifying an agent which modulates the activity of a DNA modifying enzyme the method comprising the steps of carrying out the assay of any one of Claims 16 to 23 in the presence of a test agent and comparing the fluorescence of step (ii) with that produced by the same enzyme and polynucleotide in the absence of the test agent.

25. Use of a polynucleotide according to any one of Claims 1 to 15 for assaying a DNA modifying enzyme.

26. An agent selected by the method of Claim 24 as a modulator of the activity of a DNA modifying enzyme.

27. A composition comprising an agent identifiable by the method of Claim 24 and a cancer cancer therapeutic agent.

28. A composition according to Claim 27 wherein the cancer therapeutic agent is a chemical DNA damaging agent.

29. A composition according to Claim 27 or 28 wherein there is more than one agent identifiable by the method of Claim 24, at least one of which inhibits an AP endonuclease or XPG-like enzyme and at least one which inhibits a DNA glycosylase.

30. A pharmaceutical composition comprising an agent identifiable by the method of Claim 24 and a pharmaceutically acceptable carrier.

31. A pharmaceutical composition according to Claim 30 wherein there is more than one agent identifiable by the method of Claim 24, at least one of which inhibits an AP endonuclease or XPG-like enzyme and at least one which inhibits a DNA glycosylase.

32. A pharmaceutical composition according to Claim 30 or 31 further comprising a cancer therapeutic agent.

33. An agent identified or identifiable by the method of Claim 24 or a composition as defined in any one of Claims 27 to 32 for use in medicine.

34. Use of an agent identifiable by the method of Claim 24 in the manufacture of a medicament for treating cancer.

35. Use according to Claim 34 wherein the medicament is for treatment of cancer in a patient who is administered a DNA damaging agent.

36. Use according to Claim 35 wherein the DNA damaging agent is administered prior to, at the same time as, or after administration of the agent identifiable by the method of Claim 24.

37. Use according to Claim 35 or 36 wherein the DNA damaging agent is an agent that induces the production of an AP site in DNA.

38. Use according to any one of Claims 35 to 37 wherein the DNA damaging agent is ionising radiation.

39. Use according to Claim 35, wherein the medicament is prepared from a combination of a chemical DNA damaging agent and an agent identifiable by the method of Claim 24.

40. Use of an agent identifiable by the method of Claim 24 in the manufacture of a medicament for treatment of a condition where inhibition of DNA modifying enzyme is required.

41. A method of treating cancer in a patient comprising administering to the patient an agent identifiable by the method of Claim 24.

42. A method according to Claim 41 wherein the patient is also administered a DNA damaging agent.

43. A method according to Claim 42 wherein the patient is administered the DNA damaging agent before, at the same time as, or after administration of the agent identifiable by the method of Claim 24.

44. A method according to Claim 42 or 43 wherein the DNA damaging agent is an agent that induces the production of an AP site in DNA.

45. A method according to any one of Claims 42 to 44 wherein the DNA damaging agent is ionising radiation.

46. A method of inhibiting the activity of a DNA modifying enzyme comprising contacting the enzyme with an agent identifiable by the method of Claim 24.

47. A method according to Claim 46 wherein the agent is administered to a patient who has a condition where inhibition of the activity of an AP endonuclease is desired.

48. A method according to Claim 46 wherein the agent is administered to a patient who has a condition where inhibition of the activity of a DNA glycosylase enzyme is desired.

49. A method according to Claim 46 wherein the agent is administered to a patient who has a condition where inhibition of the activity of a XPG-like enzyme is desired.

50. A therapeutic system comprising an agent identifiable by the method of Claim 24 and a cancer therapeutic agent.

51. A therapeutic system according to Claim 50 wherein the cancer therapeutic agent is a chemical DNA damaging agent.

52. A method for diagnosing and/or prognosing cancer in a patient comprising the steps of

(i) incubating a sample containing cells from the patient with a polynucleotide according to any one of Claims 1 to 8;
(ii) detecting fluorescence; and
(iii) comparing the fluorescence of step (ii) with that produced by a control sample.

53. A method to determine the sensitivity of a patient to a platinum based therapy, comprising the steps of

(i) incubating a sample containing cells from the patient with a polynucleotide according to any one of Claims 1 to 5 or 8;
(ii) detecting fluorescence; and
(iii) comparing the fluorescence of step (ii) with that produced by a control sample.

54. A method according to Claim 53 wherein the polynucleotide is a substrate for an XPG-like enzyme.

55. A method according to any one of Claims 52 to 54 wherein the sample is a sample from tissue in which cancer is found or suspected.

56. A method according to Claim 52 or 55 wherein the cancer is testicular or ovarian cancer.

57. The use of the agent identifiable by the method of Claim 24 for the manufacture of a medicament for the treatment of a microbial disease.

58. A method of detecting the mutagenic, cytostatic or cytotoxic nature of a test compound, which method comprises:

(i) preparing test cells by contacting cells with one or more agents which are inhibitors identifiable by the method of Claim 24;

(ii) in those test cells, monitoring the frequency of phenotypic change, the cell proliferation or the frequency of cell death (as appropriate) in the presence and absence of said test compound.

59. A method of assessing the ability of a compound to protect against DNA damage, which method comprises:

(i) preparing test cells by contacting cells with one or more agents which are inhibitors identifiable by the method of Claim 24;

(ii) contacting those test cells with a known carcinogen; and

(iii) monitoring the frequency of DNA damage in those test cells in the presence and absence of said compound.

60. A kit of parts comprising a polynucleotide according to any one of Claims 1 to 15 and a DNA modifying enzyme.

61. A kit of parts according to Claim 60 wherein the enzyme is an AP endonuclease.

62. A kit of parts according to Claim 60 wherein the enzyme is an XPG-like enzyme.

63. A kit of parts according to Claim 60 wherein the enzyme is a DNA glycosylase.

64. A kit of parts according to Claim 63 further comprising the HAP1 enzyme.

65. A kit of parts according to Claim 63 further comprising a chemical cleavage agent.

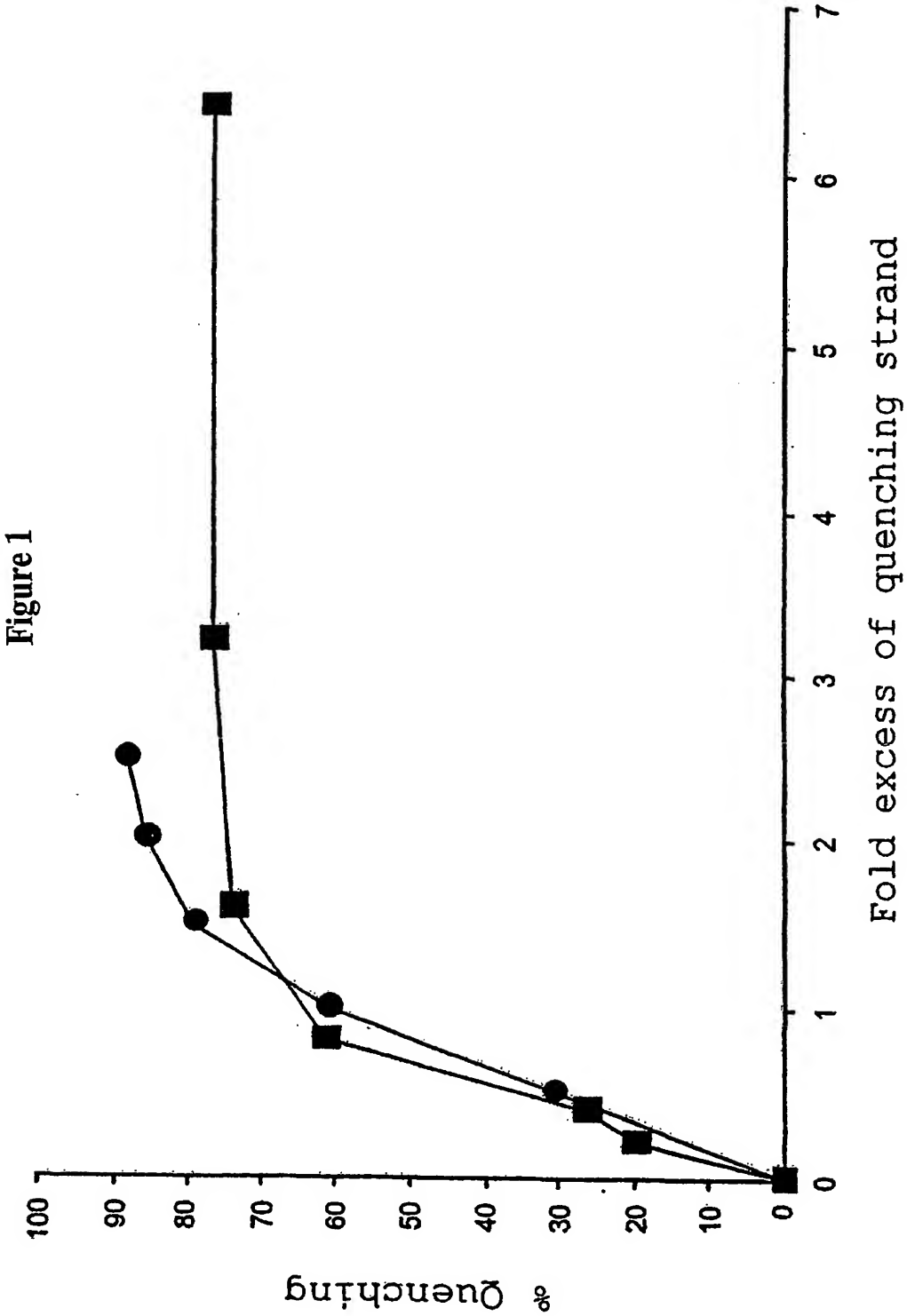
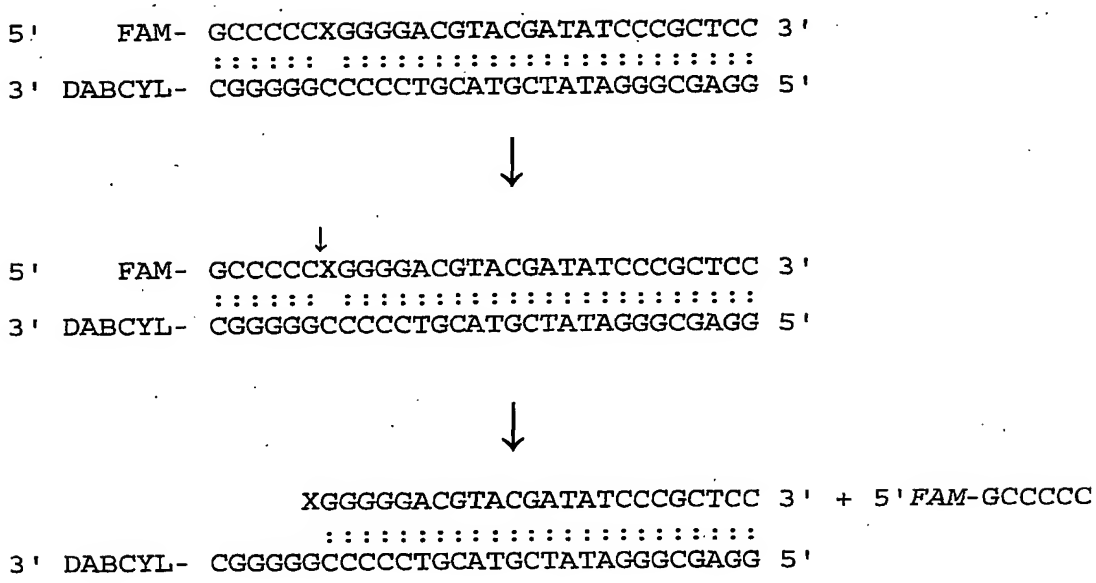

% Quenching
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
Fold excess of quenching strand

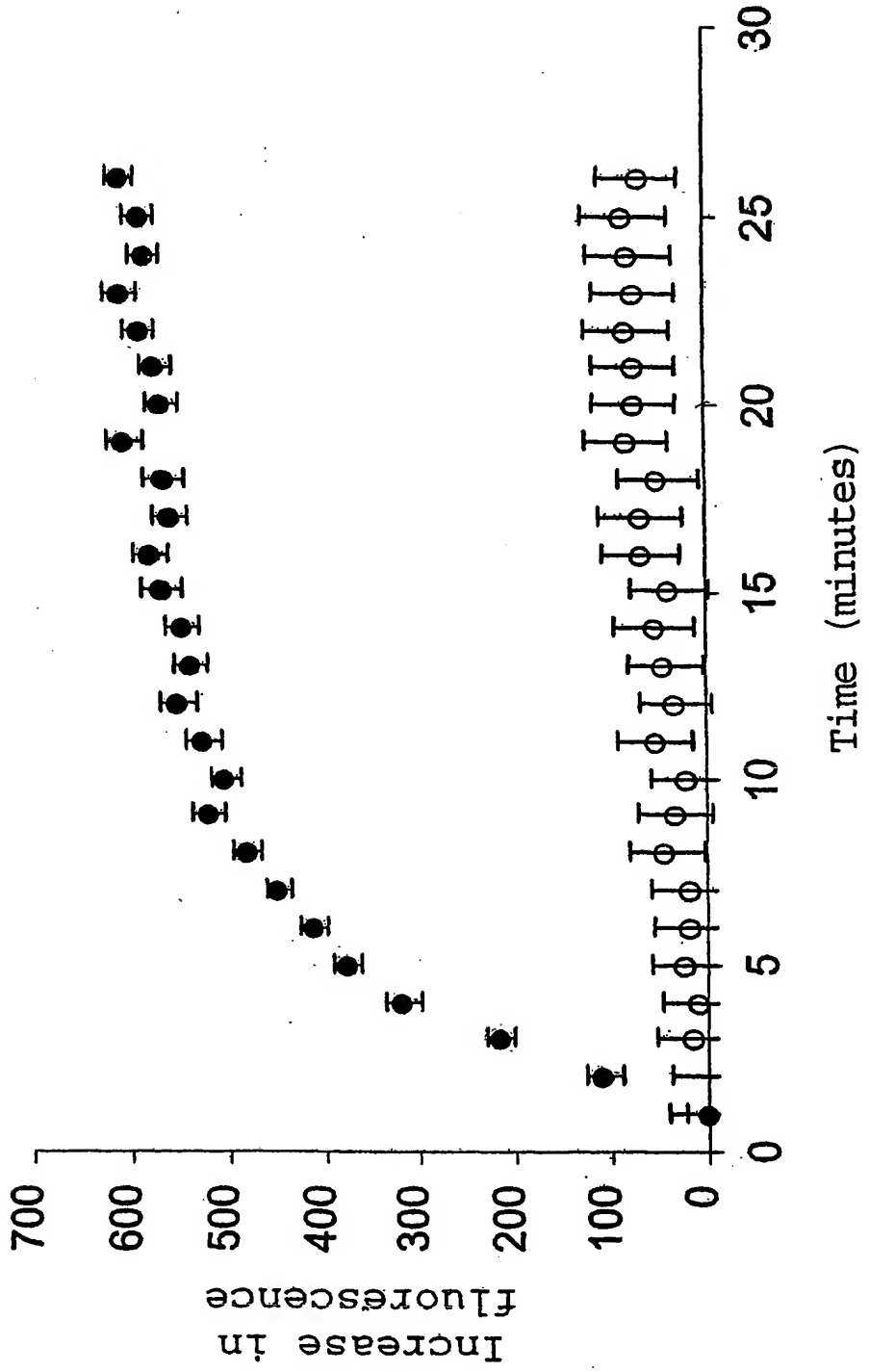
Figure 1

Figure 2



X	= an artificial 'abasic' site, (e.g. Tetrahydrofuran)
FAM	= quenched fluoroscein fluor.
FAM	= excitable fluoroscein fluor.
DABCYL	= quenching molecule
↓	= scission of the phosphodiester bond

Figure 3


Increase in fluorescence
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
Time (minutes)

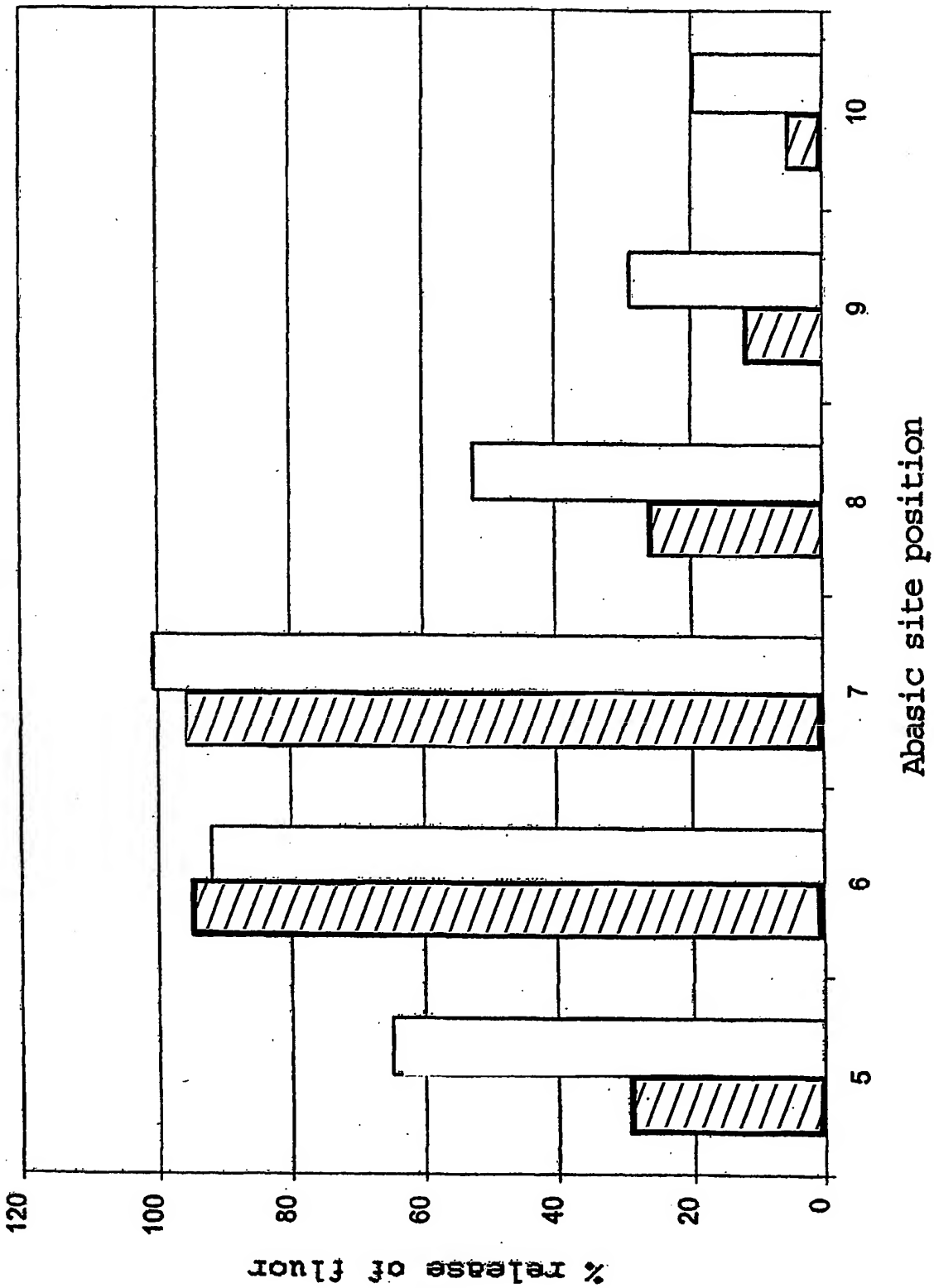

Abasic site position
10
9
8
7
6
5
% release of fluor
120
100
80
60
40
20
0

Figure 4

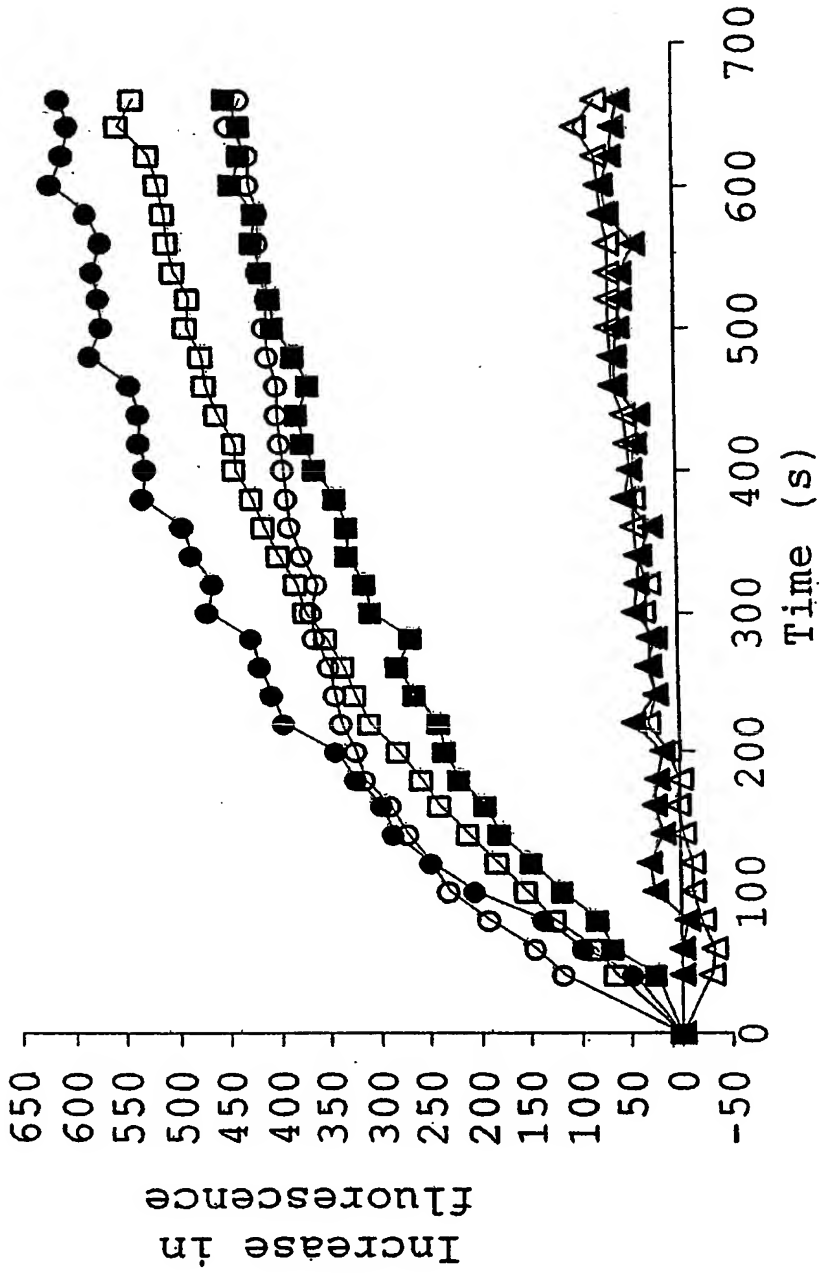
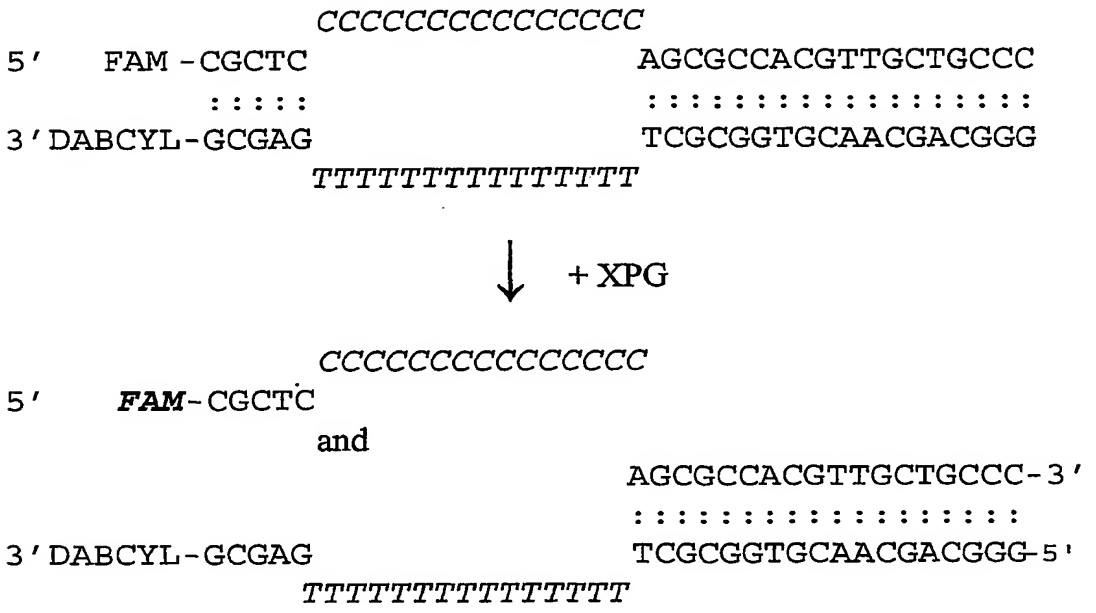

Increase in fluorescence
650
600
550
500
450
400
350
300
250
200
150
100
50
0
-50
0
100
200
300
400
500
600
700
Time (s)

Figure 5

Figure 6



Or potentially:

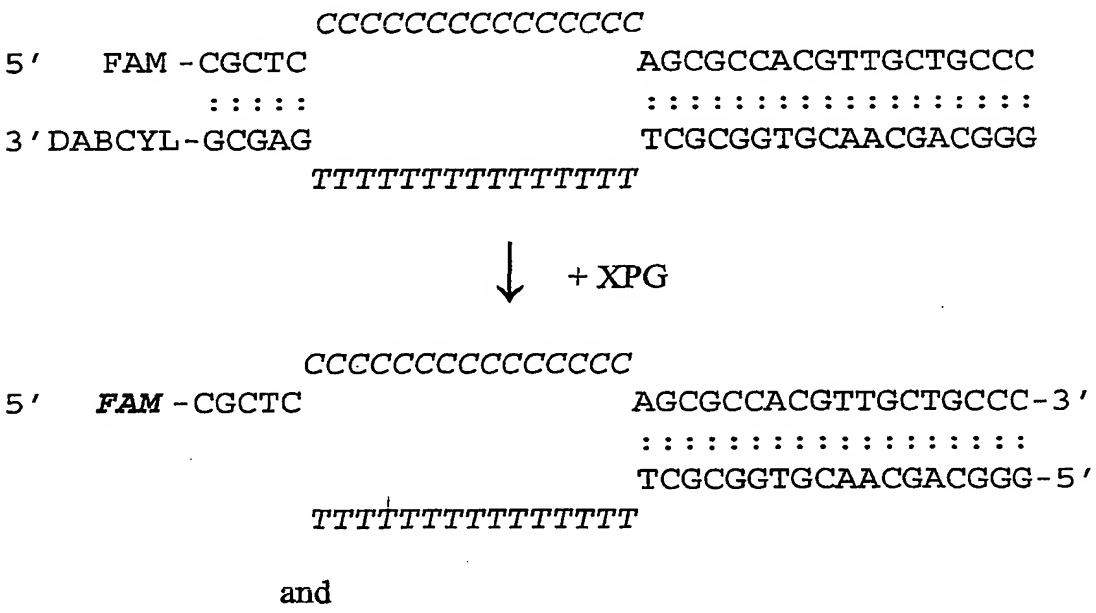
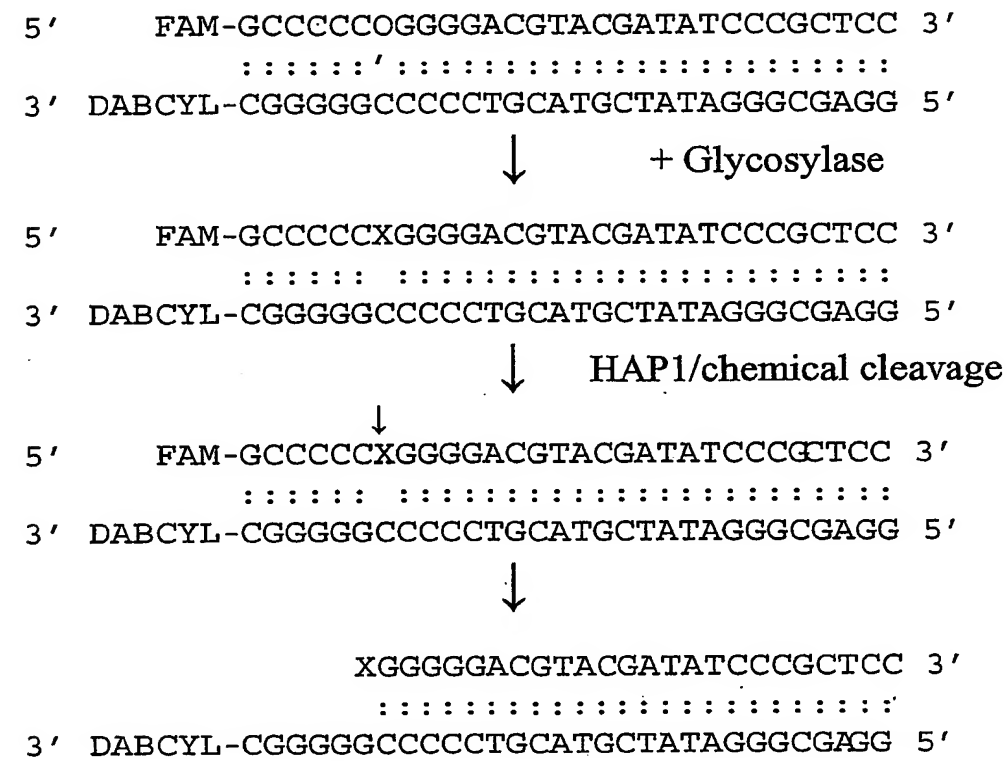


Figure 7: page 1



FAM = quenched fluoroscein fluor.
FAM = exciteable fluoroscein fluor.
DABCYL = quenching molecule

X = an artificial 'abasic' site, (e.g. Tetrahydrofuran)
O = a damaged base (e.g. 8-oxoguanine) introduced into the DNA by chemical methods
: = normal base pairing
′ = potentially altered base pairing from damaged base
↓ = scission of the phosphodiester bond

Figure 7: page 2

```
5'     FAM-GCCCCCCTATCCCGCTCCTAT 3'
           :':::':':::::::':::':::::
3' DABCYL-COGGOGOATAGGGOGAOGATA 5'
```

↓

```
5'     FAM-GCCCCCCTATCCCGCTCCTAT 3'
           :  ::  :  ::::::  ::  ::::
3' DABCYL-CXGGXGXATAGGGXGAXGATA 5'
```

↕

```
5'     FAM-GCCCCCCTATCCCGCTCCTAT 3'
```

and

```
3' DABCYL-COGGOGOATAGGGOGAOGATA 5'
```

FAM = quenched fluoroscein fluor.
FAM = exciteable fluoroscein fluor.
DABCYL = quenching molecule

X = an artificial 'abasic' site, (e.g. Tetrahydrofuran)
O = a damaged base (e.g. 8-oxoguanine) introduced into the DNA by chemical methods
: = normal base pairing
' = potentially altered base pairing from damaged base

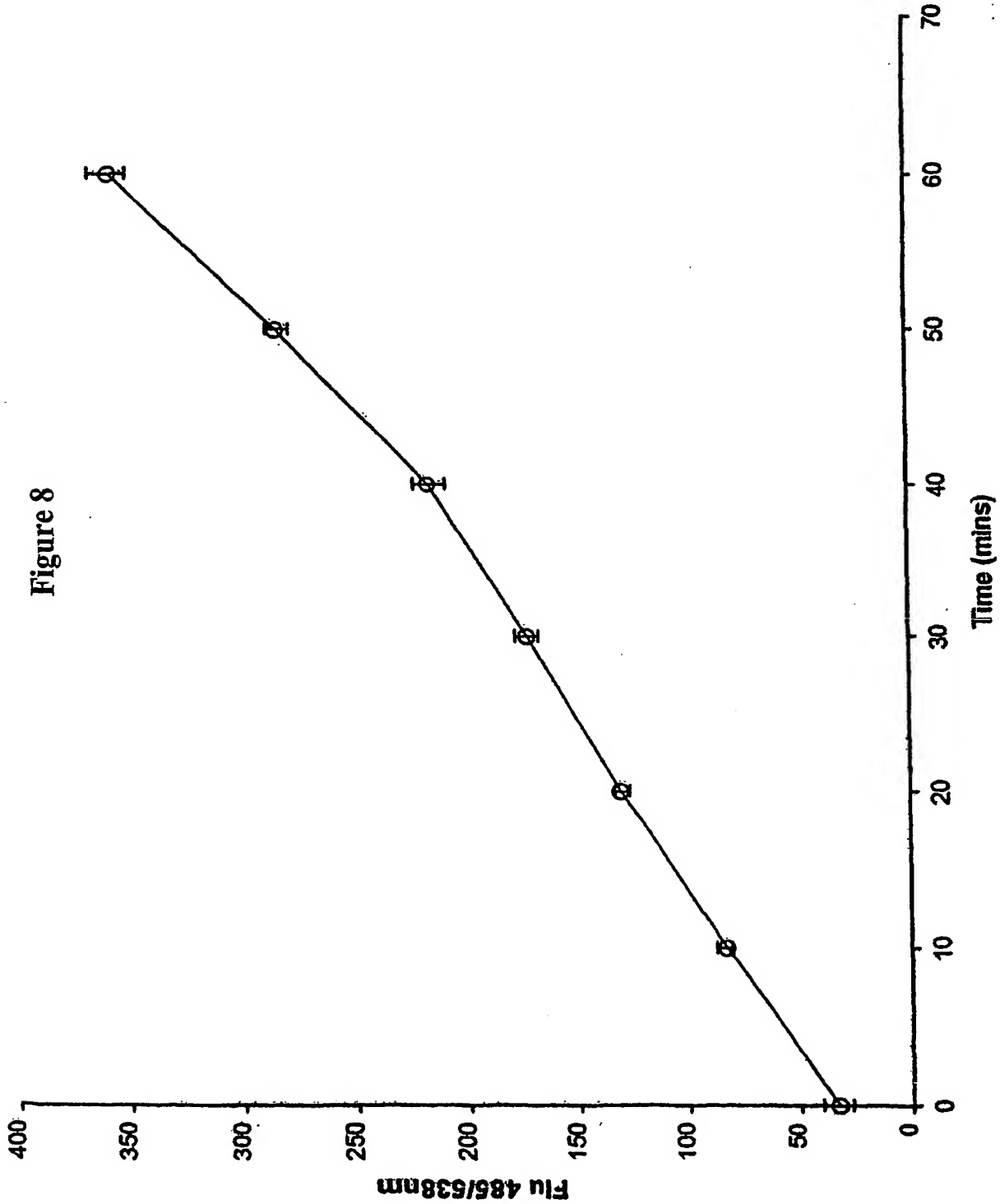

Flu 486/538nm
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
Time (mins)

Figure 8

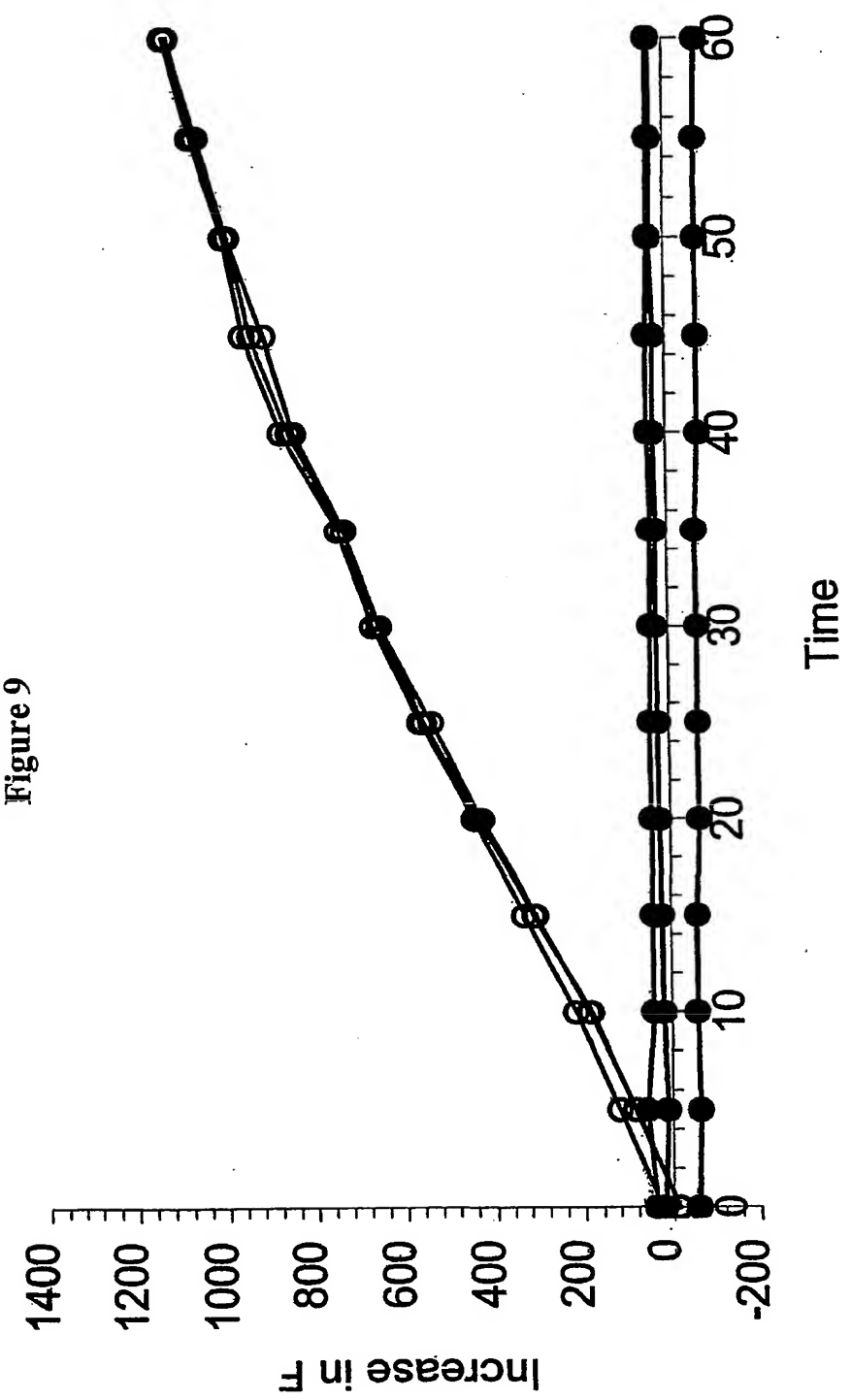

Increase in F
1400
1200
1000
800
600
400
200
0
-200
Time
0
10
20
30
40
50
60

Figure 9

Figure 10

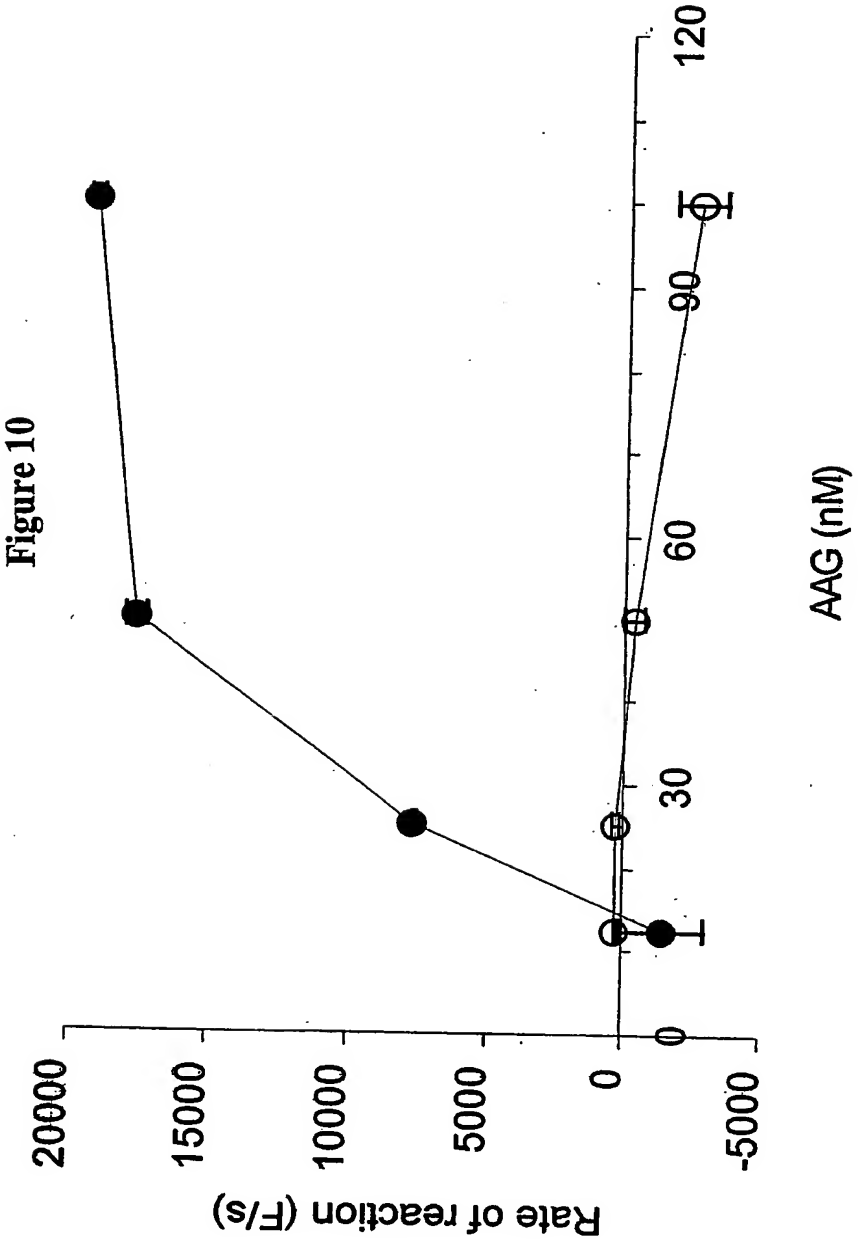
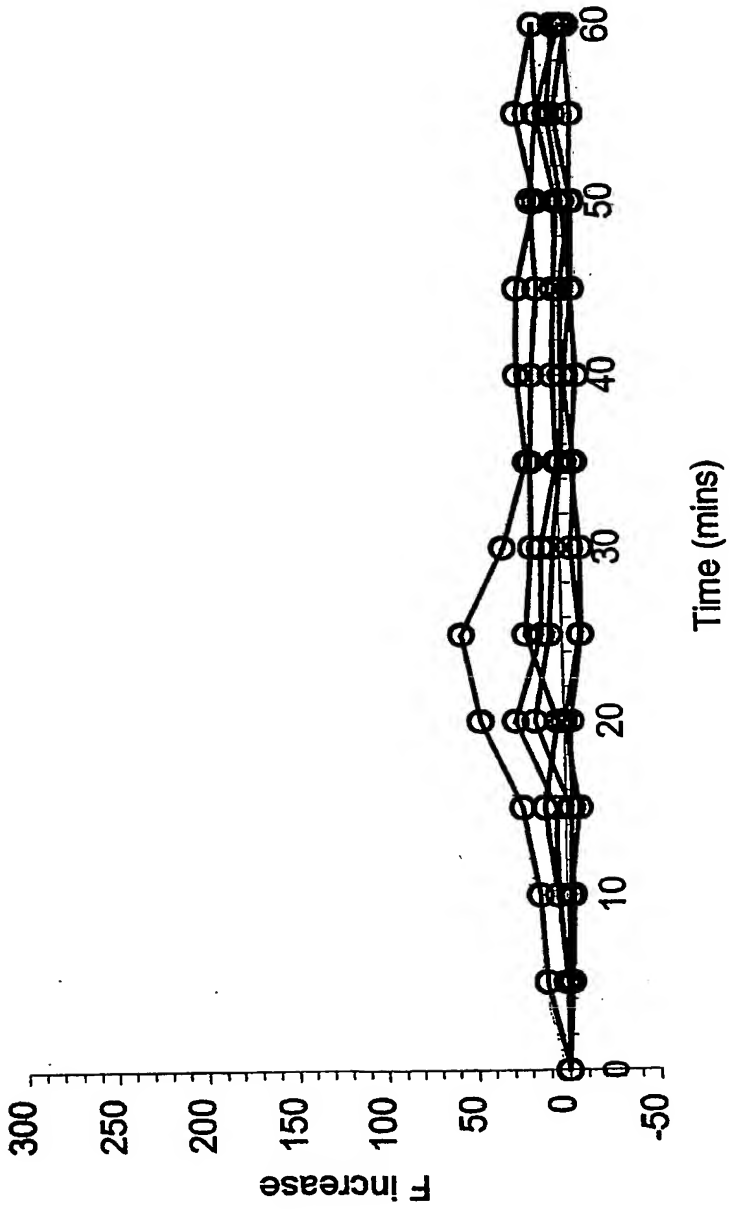

Rate of reaction (F/s)
20000
15000
10000
5000
0
-5000
0
30
60
90
120
AAG (nM)

Figure 11


F increase
300
250
200
150
100
50
0
-50
Time (mins)
0
10
20
30
40
50
60

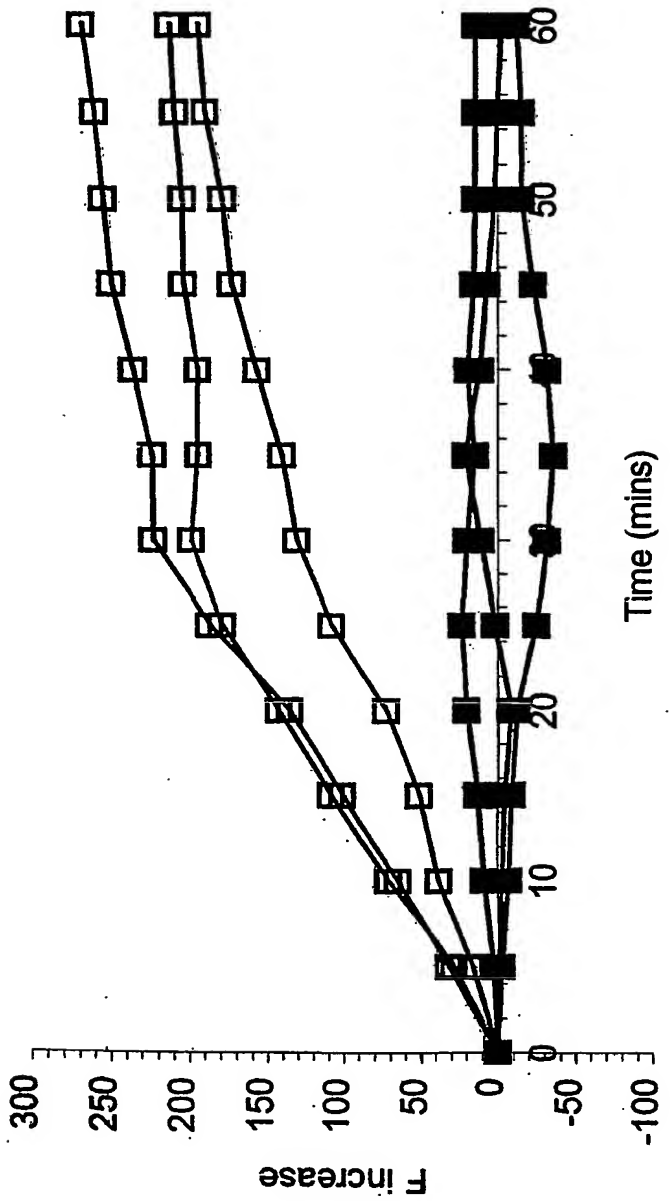


Figure 12

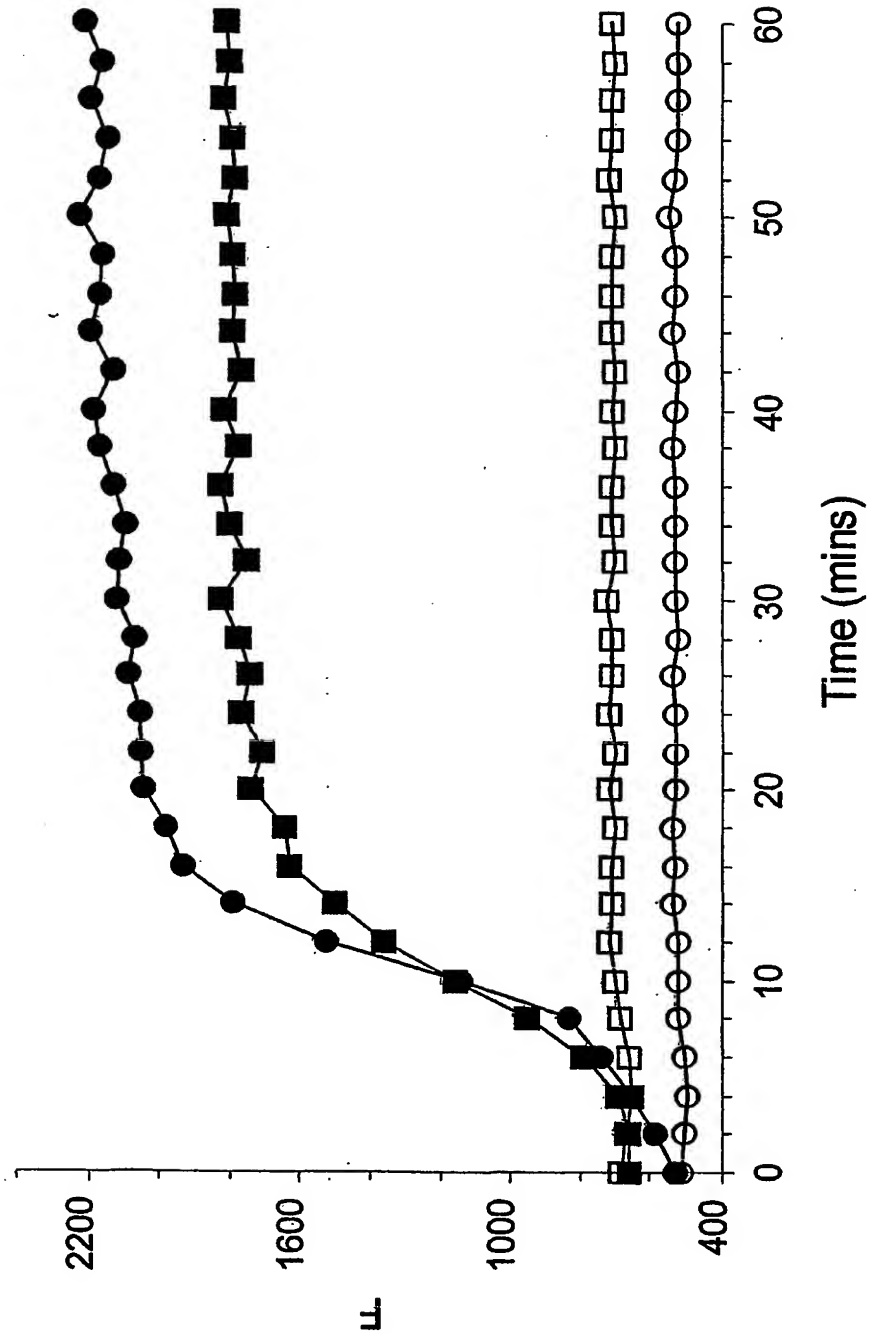


Figure 13

Figure 14

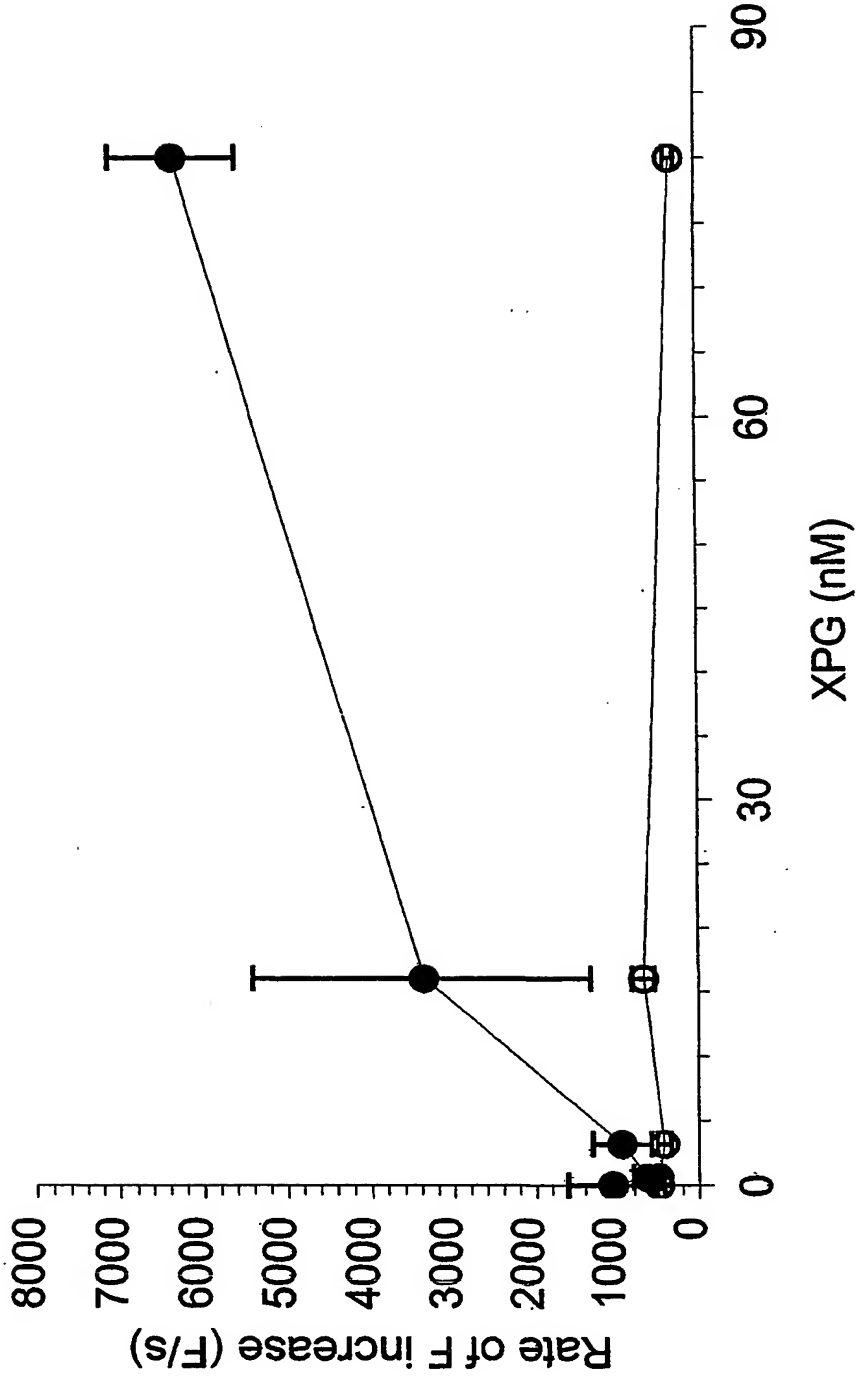
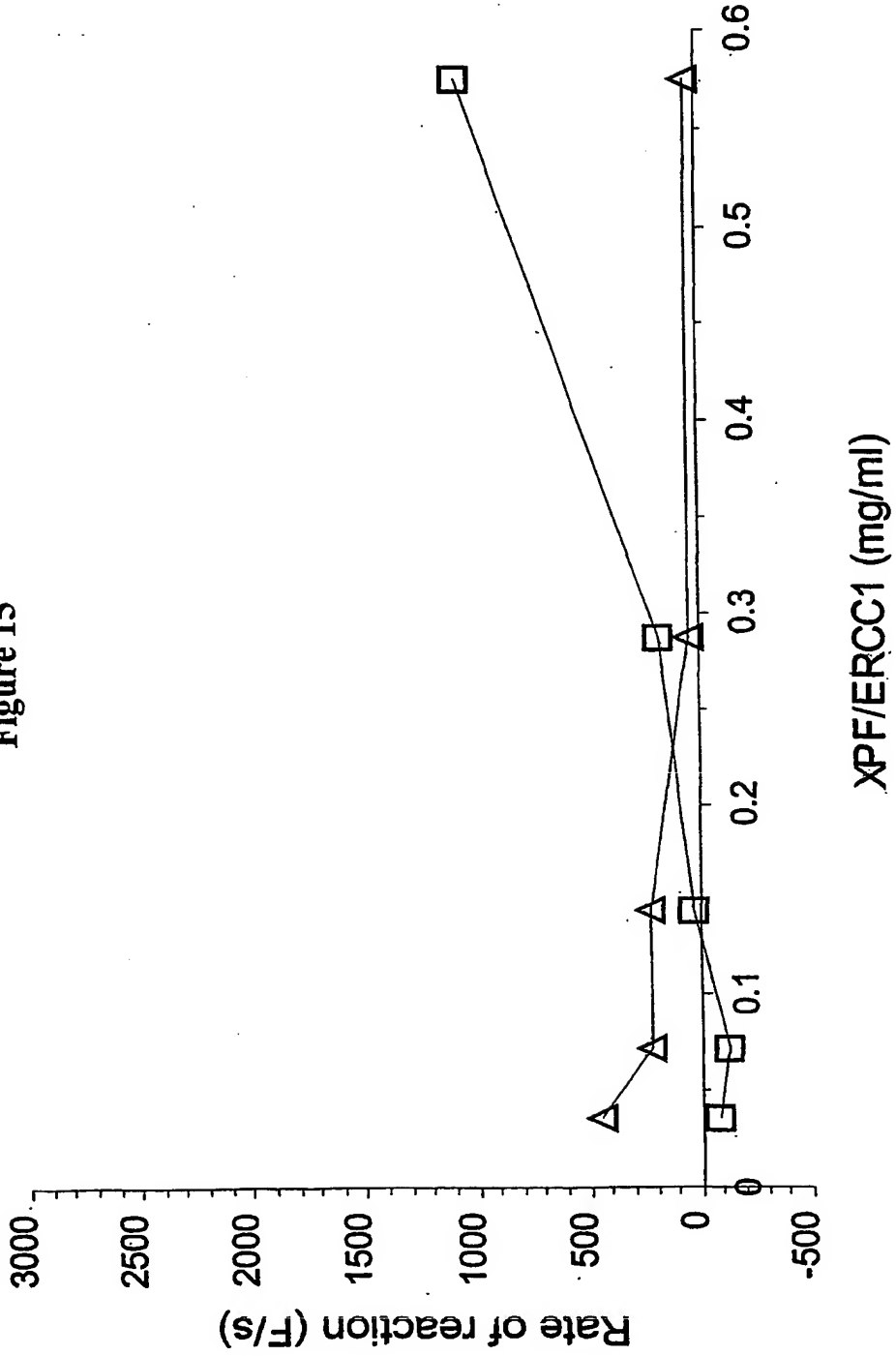

Rate of F increase (F/s)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
30
60
90
XPG (nM)

Figure 15


Rate of reaction (F/s)
3000
2500
2000
1500
1000
500
0
-500
XPF/ERCC1 (mg/ml)
0
0.1
0.2
0.3
0.4
0.5
0.6

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(74) Agent: **MILES, John, S.**; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

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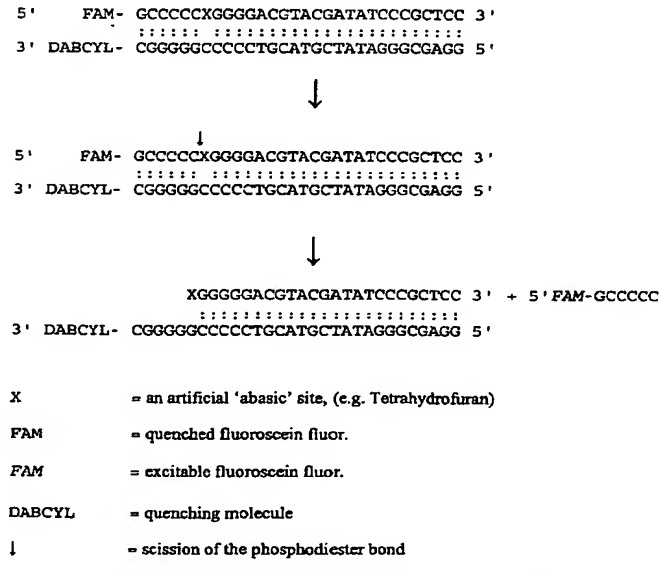
Published:
— *with international search report*

[Continued on next page]

(54) Title: METHODS AND POLYNUKLEOTIDES FOR ASSAYING THE ACTIVITY OF A DNA MODIFYING ENZYME



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FAM	= quenched fluoroscein fluor.
FAM	= excitable fluoroscein fluor.
DABCYL	= quenching molecule
↓	= scission of the phosphodiester bond

(57) Abstract: The present invention provides a polynucleotide having a double stranded portion which is interrupted by at least one residue of the polynucleotide which does not participate in an A-T or G-C base pair, the molecule further having attached thereto a fluorescent moiety and a quenching moiety which quenches the fluorescence of the fluorescent moiety. The invention further provides assays and methods using said polynucleotides, and uses of agents identifiable using said polynucleotides which inhibit DNA modifying enzymes.

WO 03/008643 A3

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
21 August 2003

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INTERNATIONAL SEARCH REPORT

Interna application No
PCT/ JJ2/03345

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C12Q1/34 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI J J ET AL: "Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA." NUCLEIC ACIDS RESEARCH. ENGLAND 1 JUN 2000, vol. 28, no. 11, 1 June 2000 (2000-06-01), page E52(I-V) XP002244017 ISSN: 1362-4962 page I -page II; figure 1 --- -/--	1,2,4,8, 11,12, 14-17, 25,60

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 June 2003	30/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Steffen, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/GB 02/03345

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TYAGI S ET AL: "MOLECULAR BEACONS: PROBES THAT FLUORESCE UPON HYBRIDIZATION" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 14, 1 March 1996 (1996-03-01), pages 303-308, XP000196024 ISSN: 1087-0156 page 307, right-hand column, paragraph 3; figures 1,2	1,2,4,8, 11-15
X	EP 0 930 370 A (CENTEON PHARMA GMBH) 21 July 1999 (1999-07-21) paragraph '0009!; figure 1 paragraph '0016! paragraph '0019!	1,3,4,8, 11-14
A	WO 99 35288 A (MINNESOTA MINING & MFG) 15 July 1999 (1999-07-15) page 7, line 24 - line 31; figure 3	

INTERNATIONAL SEARCH REPORT

Int ional application No.
CT/GB 02/03345

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 26-51, 57-59
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 26-51, 57-59

Present claims 26-51, 57-59 relate to structurally undefined products defined by a parameter or property, namely the fact to be selected or identifiable by the method as claimed in claim 24. The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, no search has been carried out for claims 26-51, 57-59, that relate to the above mentioned products.

Moreover, the claims cover all products having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search is impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

ation on patent family members

Intern application No
PCT/GB 02/03345

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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